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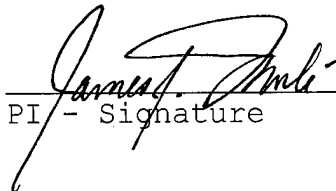
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## INTRODUCTION:

The clinical relevance of immunotherapy for the treatment of cancer is premised on the ability to generate a specific cellular immune response against tumor antigens (1). Unlike conventional treatments for tumors (chemotherapy, radiation or surgery) immunotherapy offers the ability of the patient's own immune system to retard tumor growth. However, the weak immunogenicity of some tumors, induction of tolerance by the tumor (2), and the risk of autoimmunity hamper the clinical efficacy of immunotherapy. Some of the treatment regimens designed to elicit anti-tumor immunity include the administration of immunostimulatory cytokines (3, 4), adoptive transfer of in vitro stimulated tumor-reactive T cells (5-8), and administration of antibodies against tumor-associated antigens (9, 10).

Therapeutic cancer vaccines targeted against tumor-associated antigens represent another approach to immunotherapy. Two necessary properties involved in generating tumor vaccines are the presence of immunodominant epitopes expressed by tumors and the presentation of tumor-associated antigens by professional APC (11, 12). Presentation of these antigens on MHC class I or class II molecules by APC expressing co-stimulatory molecules such as B7 and ICAM-1, can initiate an antigen-specific T cell response which, potentially, leads to tumor rejection. Vaccines against defined tumor antigens have been formulated using purified peptides in adjuvant, recombinant viral and bacteria preparations and naked DNA vectors (13). While each of these vaccine approaches have unique properties that make them attractive candidates as therapeutics, they all have the potential problem of limiting the primary immune response against a single tumor antigen. If tumor cells down-regulate expression of the protein used as the immunogen, activated T-cells may be unable to recognize and remove those tumor cells. Another potential problem of these vaccine strategies is the efficacy of antigen uptake and presentation by APC.

Dendritic Cells (DC) are potent APC capable of eliciting a primary immune response to foreign antigens (14, 15). DC strongly express both MHC class I and class II molecules as well as the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2). DC are capable of efficient antigen uptake and priming of naïve CD4 and CD8 T-cells. Large numbers of DC can be readily cultured in vitro from precursors present in bone marrow, blood, and peripheral lymphoid organs (16-18). Because of these properties, DC are attractive candidates for use as APC in therapeutic tumor vaccines.

Preclinical research in our laboratory (19, 20) and in others (21-23) has shown that mice immunized with tumor lysate-pulsed DC (TP-DC) are protected from a subsequent challenge with a lethal dose of viable tumor cells. DC pulsed with lysates from MCA-207 fibrosarcomas are able to generate a tumor-specific cytotoxic T-lymphocyte (CTL) response in vivo (19). Immunization of either BALB/c or C57BL/6 mice with syngeneic DC pulsed with lysates from either MT-901 mammary carcinoma or MCA-207 fibrosarcomas, respectively, resulted in protective immunity from tumor challenge (19). In a therapeutic setting, TP-DC could retard the growth of established subcutaneous breast tumors and fibrosarcomas, although complete remission did not occur (19). Systemic administration of IL-2 along with the TP-DC, however, resulted in dramatic anti-tumor responses in mice harboring either subcutaneous or metastatic tumors (20). These studies suggest that the presentation of tumor antigens by DC, in the presence of immunostimulatory cytokines, results in clinically successful treatment of established tumors.

Initial clinical trials involving DC-based immunization of patients with tumors of hematologic and solid origin are promising: subjects show increased antitumor T cell reactivity and experience partial or complete clinical responses (24-29). In one study, treatment of melanoma patients with autologous DC loaded with either tumor lysates or peptide fragments of tumor antigens, resulted tumor specific immunity in 11 of 16 patients and a positive clinical response in 6 of 16 patients (2 complete remissions) (24). In a pilot study of B cell lymphoma, 3 of 4 patients receiving infusions of DC pulsed with antigen (idiotype of tumor Ig) showed an anti-tumor response (2 complete remissions) (25). In a larger lymphoma study by the same group

(26), 20 of 41 patients treated with the DC vaccine showed an antitumor response with 2 complete remissions. There was no evidence of autoimmunity in patients involved in either the melanoma or lymphoma trials. The results from both of these clinical trials suggest that the response to a DC vaccine, while promising, does not result in successful outcomes in the majority of patients. Diminished clinical efficacy may be due in part to incomplete trafficking of DC to peripheral lymphoid organs. Following s.c. immunization, the majority of DC remain in the injection site and do not traffic to draining lymph nodes or spleen, suggesting that a large number of DC may not be involved in priming a T cell antitumor response (30). It is likely that modification of DC-based vaccines resulting in increased T cell/DC interaction will result in improved clinical outcomes.

Chemokines are a growing family of small cytokines with potent chemotactic activity towards effector cells of the immune system, which are essential for leukocyte trafficking and inflammatory processes (31). These molecules are divided into 4 classes - C, CC, CXC and CX<sub>3</sub>C - based on the position of conserved cysteine residues in their sequences and bind to 7 transmembrane receptors on the surface of target cells. Chemokines have been shown to elicit or improve an antitumor response. In the 3LL lung carcinoma model in C57BL/6 mice, DC pulsed with a peptide fragment of Mut1 provided protective immunity to a lethal tumor challenge only when the DC were previously transduced with an adenoviral vector coding for the C chemokine Lymphotactin (Lptn) (21). In a therapeutic setting, Mut1-pulsed Lptn-DC were able to markedly reduce pulmonary metastases, as measured by total lung weight; however, treatment did not lead to complete remission (21). In BALB/c mice, growth of A20 leukemia was severely inhibited when these tumor cells were admixed with Lptn transfected fibroblasts but not vector transfected fibroblasts (32). Furthermore, co-expression of IL-2 along with Lptn increased the antitumor response in A20 challenged mice. Expression of IP-10, a CXC chemokine, by either J558L plasmocytoma or K485 mammary carcinoma tumor cells results in the inability of those tumors to form solid masses in vivo (33). More recently, linkage of either IP-10 or MCP-3, a CC chemokine, to a non-immunogenic tumor antigen (lymphoma idiotype Ig) elicits protective immunity in response to subsequent tumor challenge (34). Work in our own laboratory has shown that MCA-205 fibrosarcoma cells modified to express the CC chemokine RANTES were unable to form solid tumors in vivo (37). Tumor growth by RANTES expressing cells were restored by depletion of either CD8<sup>+</sup> T cells or macrophages in the host animal suggesting that the antitumor response was mediated by these cell types (35). This study represented the first analysis of the functions of RANTES as produced from an in vivo source, and showed that the chemoattractant properties of this chemokine for monocytes and T cells as predicted from in vitro assays using human cells appeared to be broadly relevant in this in vivo murine model. Taken together this suggestive body of literature implicates an active role for chemokines such as RANTES and Lptn in a therapeutic setting for cancer vaccines, possibly as a mediator of T cell/APC interaction.

Recent evidence suggests that certain specific chemokines play an important role in DC priming of T cells in vivo. Mature DC, those that are most effective at priming a T cell response, and naïve T cells express the chemokine receptor CCR7 (36,37). CCR7 is the cognate receptor for both ELC (also called MIP-3 $\beta$ ) and SLC (also called 6-Ckine), both of which are expressed predominantly in peripheral lymphoid organs (38,39). Mice deficient in expression of both SLC and ELC have diminished numbers of T cells and DC in lymph nodes (but not in spleen) and reduced trafficking of activated DC to the draining lymph node (40). Furthermore, mature DC express chemokines that can attract both naïve and recently activated T cells (41,42). It is likely that SLC (and ELC) play important roles in T cell priming by promoting DC/T cell interaction in the lymph node and spleen.

Given this background, this funded research proposal focuses on a series of studies to determine whether molecules potentially and selectively chemotactic for naïve and memory T cell subsets can be used in conjunction with tumor-pulsed DC to provide a highly effective means of both detecting and augmenting the immune response to breast cancer.

## **BODY:**

The following Technical Objectives and their corresponding timelines were specified in the original funded grant application:

1. To evaluate the capacity of human dendritic cells to detect T cell specific responses to autologous breast tumor in vitro (Months 1-48).
2. To generate high, stable chemokine producer cells by the introduction and expression of the relevant genes in human fibroblast preparations (Months 1-36).
3. To determine the capacity of the combination of chemokine-secreting cells and dendritic cells pulsed with autologous breast tumor to detect, attract, and augment specific, antigen-reactive T cells in vitro (Months 12-48).

**Note: All appended publications specifically acknowledge the support of this USAMRMC grant award (DAMD17-96-1-6103) as well as its supplemental training grant (DAAG55-97-1-0239).**

### **A. Brief Summary of Results Reported in the First (Year 1) Annual Report:**

1. We conducted studies to best generate "cytokine-driven" (GM-CSF, IL-4, and TNF- $\alpha$ ) human DC obtained from peripheral blood mononuclear cells (PBMC) as measured by yield, purity, phenotype, and function as originally proposed in Technical Objective 1.
2. We demonstrated that autologous DC could be successfully derived from PBMC of advanced breast cancer patients, which, upon antigen pulsing, could stimulate potent tetanus toxoid- and KLH-specific proliferative responses by purified T cells obtained from these same patients.
3. The above findings considerably lessened the potential concern of a compromised immune system in advanced cancer patients (as a result of multiple chemotherapy/radiation therapy regimens that are also immunosuppressive) hampering attempts to clinically develop and utilize dendritic cell-based vaccines in our proposed breast tumor immunization approaches.
4. We have successfully expressed three (3) distinct chemokine cDNAs (i.e. RANTES, lymphotactin, MIP-1 $\beta$ ) constructed into the MFG-based retroviral vector as originally proposed in Technical Objective 2. Moreover, transduced fibroblasts produced significant levels of biologically active chemokine(s) by the introduced transgene(s).

### **B. Brief Summary of Results Reported in the Second (Year 2) Annual Report:**

1. In Technical Objective 1, we demonstrated that the addition of TNF- $\alpha$  to GM-CSF and IL-4 (at a critical timepoint of culture) resulted in both a significantly greater yield (at least two-fold) of DC and enhanced antigen presenting function.
2. In Technical Objective 1, we pursued a new strategy to generate CTL that utilized known HER2/neu and CEA peptides, which have been reported to elicit specific T cell reactivity in breast cancer patients.

3. In Technical Objective 2, we identified new chemokines (i.e. SLC/6Ckine and ELC) that have been shown to be highly selective in their recruitment of DC and/or T cell subsets (see also new data presented below).

4. In Technical Objective 2, using a bioloistics device ("gene gun"; BioRad), we were highly successful in obtaining short-term lines of fibroblasts from breast cancer patients that secrete high levels of the transgene encoded chemokines for at least 7-10 days in culture.

5. In response to the issues raised in the original Peer Review Panel report, we embarked on murine studies utilizing a syngeneic breast tumor (denoted MT-901).

6. We optimized the generation of murine DC from spleen and bone marrow.

7. We demonstrated that immunizations with tumor lysate-pulsed DC can mediate effective immune priming in vivo and can successfully treat established visceral lung metastases from the MT-901 breast carcinoma.

8. We embarked on additional human studies with direct relevance to breast cancer patients. We were highly successful in obtaining both DC and CD34<sup>+</sup> hematopoietic stem/progenitor cells with potent functional activity from the same leukapheresis collects from G-CSF-primed patients in sufficient numbers for the future purpose of combining peripheral blood stem cell transplantation with DC-based immunization strategies for the treatment of breast cancer.

### **C. Brief Summary of Results Reported in the Third (Year 3) Annual Report:**

1. In Technical Objective 1, using DC generated under fully optimized culture conditions, we were capable of reliably eliciting potent and specific T cell responses to known "model" antigens. As example, human DC derived from PBMC of an HLA-A2<sup>+</sup> breast cancer patient cultured in GM-CSF and IL-4 in serum-free medium were able to potently stimulate autologous T cells to the defined antigen, KLH, which in all cases have surpassed the robust stimulatory capacity of an optimal concentration of the lectin, PHA.

2. In Technical Objective 2, we focused our efforts on two new chemokines SLC and ELC. We showed that both SLC and ELC could mediate rapid and potent recruitment of naive T cells and, importantly, also DC as measured by 2-hour chemotaxis assays in vitro.

3. In Technical Objective 2, the introduction of 6Ckine (i.e. SLC) in vivo in mice resulted in the selective recruitment into the skin site of host-derived CD3<sup>+</sup> T cells comprised of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Of importance, the number of recruited naive T cells harvested from the site of chemokine gene introduction was substantial ( $>18 \times 10^6$  per centimeter diameter of excised and processed skin).

4. We made the important discovery in our murine tumor models that the systemic administration of non-toxic doses of interleukin-2 (IL-2) could potentiate the antitumor effects of tumor-pulsed DC in vivo during both primary immunization and treatment of established tumors. Further, IL-2 could enhance CTL activity and IFN- $\gamma$  production in the treated animals. These findings have resulted in our plans to initiate human clinical trials in breast cancer patients.

5. We showed that direct intratumoral injections of "unpulsed" (i.e. not tumor antigen-loaded or charged) DC could mediate the regression of established breast tumor nodules in mice. We have further showed that antitumor efficacy appears to be correlated with the level of baseline apoptosis measured within the tumor mass before the local delivery of DC. Of



importance, tumor nodules at distant sites from the DC-injected tumor nodule also underwent regression. The immunotherapeutic effect elicited by intratumoral injections of DC in this murine breast tumor model was critically dependent upon potent activation of a host-derived T cell immune response, both locally and systemically.

6. We also showed that pulsing DC with KLH before in vivo injection can markedly augment their antitumor effect in the MT-901 breast tumor model, which could be enhanced further by the systemic administration of IL-2.

7. We were awarded an R01 grant from the NCI-NIH based on our data generated during this third year of the DOD project, entitled "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer". Of importance, this funded grant involves a clinical trial, entitled: "A Phase II Study of Direct Tumor Injection of KLH-Pulsed Autologous Dendritic Cells in Patients with Metastatic Breast Cancer", which received approval from our cancer center review board, IRBMED, and the FDA to proceed.

#### **D. Results for the Current (Year 4) Annual Report:**

The research conducted during the fourth year of this four-year award continued to concentrate mainly on the preclinical breast tumor studies in mice. **This latter effort continues to result in important new data (see below), leading to the initiation of one phase II clinical trial in advanced breast cancer patients and a second clinical trial proposed for the chemokine SLC. In addition to this clinical effort, the studies conducted under this award in year 4 have resulted in an additional five full-length publications (also appended) and four abstracts.** All data figures and tables referred to in the text below are provided in the Appendix section of this annual report.

(a) Technical Objective 1: **Our efforts in this aim are completed.** In previous annual reports we successfully optimized the generation/production of human dendritic cells (DC) to serve as potent antigen presenting cells (APC) in order to best detect and enhance low level specific T cell responses in vitro. Using DC generated under fully optimized culture conditions, we were capable of reliably eliciting potent and specific T cell responses to known "model" antigens as well as Her2/neu-encoded peptide.

More recently, we have evaluated the capacity of monocyte conditioned medium (MCM) to mature DC in the presence of GM-CSF and IL-4. The addition of MCM elicited maturation of human PBMC-derived DC, as measured by increases in the expression of CD83, CD80, and CD86 (data not shown). In every case, however, the exposure of human DC to the human recombinant trimeric form of CD40L resulted in the most pronounced increases in costimulatory molecules as well as CD83 expression on the resultant mature DC as measured by FACS analysis (Figure 1). The use of CD40L to mature DC has now been incorporated into our production of potent human DC for our clinical trials.

(b) Technical Objectives 2 and 3 (Preclinical): **Our efforts in these aims are now near completion.** Using our MT-901 breast tumor model in BALB/c mice, we have now been successful in reducing the growth of established tumor by the local delivery of the chemokine SLC. We found that intratumoral injections of SLC (on days 7-9) significantly reduced the size of the breast tumor, while subcutaneous injection of SLC at a distant site had no significant antitumor effect ( $P > 0.01$ ; Figure 2). **To our knowledge, this finding represents the first evidence of SLC reducing breast tumor growth in vivo.**

We have now been successful in introducing the chemokine gene encoding for SLC directly into DC rather than using primary fibroblasts. The power of this strategy allows potent antigen presenting DC to also serve as the source of local SLC production at the vaccine site to elicit an enhanced local immune response. In collaboration with Dr. Jeffrey Chamberlain's

laboratory, we have successfully generated a "gutted" recombinant adenoviral vector, denoted aD2028#16 (Ad-SLC), which carries an SLC expression cassette in its E1 region. Figure 3 provides diagrams for both the SLC gene-containing and control GFP gene-containing Ad vector constructs. A cassette was excised as an SfiI-BspLU11I fragment from pCMVII-Amp-SLC, blunt-ended, and cloned into the BglII site of shuttle vector pD1954-BglII. The resulting plasmid contains Ad DNA from 0-1, 9.3-20.2, and 98.2-100 map units. This plasmid was digested with BspEI to separate the left and right ends of the Ad genome and recombined in BJ5183 cells with Hirt prep DNA prepared from mammalian cells infected with an E1-, E3-deleted adenovirus. The intact Ad-SLC genome was released from the resulting plasmid (pD2028#16) by restriction digest and transfected into C7 cells to recover virus. pAdEasy1-GFP, containing the Ad-GFP genome, was a gift from Dr. Bert Vogelstein. Viruses were propagated on C7-cell monolayers and purified on CsCl gradients according to a standard protocol. Purified virus was dialyzed against 20 mM HEPES (pH 7.4) containing 5% sucrose, aliquoted, and frozen in a dry ice/ethanol bath. OD260 was determined after particle disruption at 56C for 10 min. in 0.1% SDS, 10 mM Tris-Cl (pH 7.4), and 1mM EDTA. Particle concentration was calculated using an extinction coefficient of  $9.09 \times 10^{13}$  OD ml cm virion<sup>-1</sup>. Plaque assays were also performed and yielded similar vector particle to infectious unit ratios for all preparations (mean  $X \pm Y$ ).

For adenovirus infection, murine and human DC (generated by culturing murine bone marrow cells or human peripheral blood monocytes in GM-CSF and IL-4 for 7 days) were resuspended at a concentration of  $10^7$  ml<sup>-1</sup> in reduced-serum, complete medium and placed in a 15-ml conical tube. Virus was added, the suspension was mixed well, and the tube was incubated at 37C for 2-4 hr. Nine volumes of complete medium were then added and the DC were transferred to tissue culture dishes. Comparisons were also made between infection with a range (e.g., 5,000, 16,000, and 25,000) of transducing units per DC. Supernates were collected and tested for SLC activity by microchemotaxis assay. As measured by GFP expression by FACS, 40% and 60% of murine and human DC, respectively, could be successfully infected at 5,000 T.U. per DC; greater percentages of DC infected by Adv-GFP were obtained at higher T.U. per DC (data not shown). The percentages of migrating cells in response to the SLC-containing supernate in microchemotaxis assays was compared against a standard curve of recombinant SLC protein to determine concentration. Figure 4 shows a representative experiment. As measured by the chemotaxis of fresh, naïve murine T cells, SLC produced by  $1 \times 10^6$  Adv-infected murine DC within 24 hr. approached 2  $\mu$ g and remained  $>1 \mu$ g after 3 days post-infection. Moreover, the level of SLC produced by the  $1 \times 10^6$  Adv-infected murine DC was far superior to that produced by DC transfected with an SLC-expression plasmid via the FuGENE 6 reagent. Adv-SLC-infected (but not control, Adv-GFP-infected) murine DC also reproducibly produced SLC as measured by chemoattraction of additional DC in microchemotaxis assays (Figure 5). Of importance, the phenotypic profile of murine DC remained unchanged following infection with the Adv-SLC vector, including the expression of CD40, CD80, CD86, CD11b, CD11c, MHC class I and MHC class II (data not shown).

The in vivo injection of SLC-producing DC could not only inhibit tumor growth but could also mediate the potent migration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to the vaccine injection site. Figure 6 shows a representative FACS analysis of enzymatically dispersed skin biopsies taken from the injected animals. We found that SLC producing DC attracted ~2-3 fold more CD4 and CD8 T cells to the injection site at 3 days post-injection. These data suggest that the improved adjuvanticity and antitumor effect of DC resulting from the production of SLC may be due to the increased migration of T cells to the site of immunization.

Overall, similar results were observed in vitro with human DC. Figure 7 shows that SLC production approximated 1.5  $\mu$ g from  $1 \times 10^6$  human DC infected by the Adv-SLC vector construct, as measured by the recruitment of fresh, naïve unfractionated, CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The phenotypic profile of human DC also remained unchanged following Adv-SLC infection (data not shown). These data position us to initiate a clinical trial in advanced breast cancer patients of SLC-producing human DC as a follow-up to our recently initiated clinical trial of intratumoral DC injections (see below).

**(b) Technical Objectives 2 and 3 (Clinical):** Because of our preclinical results provided in our previous (year 3) report, we submitted an R01 grant proposal to the NCI-NIH entitled "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer". This grant received an outstanding score and was awarded full funding. The proposal involves translation of proposed preclinical murine studies to well-defined phase II clinical trials in patients with advanced breast cancer. A draft clinical protocol was provided in our previous (year 3) report, entitled: "A Phase II Study of Direct Tumor Injection of KLH-Pulsed Autologous Dendritic Cells in Patients with Metastatic Breast Cancer". **This trial has now been initiated after receiving full approval from both our IRBMED and the FDA (under an IND).** Two patients have completed treatment. As detailed in the clinical protocol, each patient received intratumoral injections of  $1 \times 10^7$  KLH-pulsed DC, three times, one week apart. The site of injection in both patients was a tumor-involved axillary lymph node. The two patients are now in follow-up. Pre- and post-treatment biopsies of the lymph node have been taken and are currently being evaluated for level of apoptosis (by TUNEL assay) and immune cell infiltrates (by immunohistochemistry). No adverse, treatment-related side effects was noted. Accrual of additional patients is currently underway.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- We have optimized the generation of dendritic cells from the peripheral blood of advanced breast cancer patients.
- We have shown that antigen-pulsed human dendritic cells can generate potent and specific T cell responses to both "standard" (KLH, TT) antigens and breast cancer-related peptides (e.g., HER2/neu) in vitro.
- We have identified chemokines (e.g., SLC/6Ckine, ELC) that can selectively recruit both naive T cells and dendritic cells.
- We have shown in preclinical animal studies that the systemic administration of non-toxic doses of IL-2 can markedly enhance the efficacy of dendritic cell-based cancer vaccines.
- We have discovered in preclinical animal studies a potentially important link between breast tumor apoptosis and susceptibility to treatment by dendritic cells.
- The chemokine SLC has potent antitumor activity in vivo in preclinical animal studies.
- We have successfully generated a recombinant adenovirus vector containing the SLC gene, which can transduce dendritic cells at high efficiency
- In preclinical animal studies, we have shown that SLC gene-modified dendritic cells can reliably produce large amounts of the chemokine and selectively recruit T cells and other dendritic cells to the site of injection in vivo.
- We have initiated a phase II clinical trial of the direct intratumoral injection of dendritic cells into accessible nodules in patients with advanced breast cancer

#### **REPORTABLE OUTCOMES:**

- A. Publications: (10 through 17 represent the progress in this current annual report)**

1. Chen, B.-G., Smith, J.D., Geiger, J.D., and Mulé, J.J.: TNF-alpha expands human dendritic cells cultured in serum-free medium and enhances soluble antigen presentation to CD4<sup>+</sup> T cells. J. Allergy Clin. Immunol. 99:S450, 1997.
2. Choi, D., Walsh, M., Hoffmann, S., Ratanatharathorn, V., Uberi, J., Chang, A., McDonagh, K.T., and Mulé, J.J.: Dendritic cell-based vaccines in the setting of peripheral blood stem cell transplantation: CD34<sup>+</sup> cell-depleted mobilized peripheral blood can serve as a source of potent dendritic cells. Blood 90: 538a, 1997.
3. Fields, R.C., Osterholzer, J.J., Fuller, J.A., Thomas, E.K., Geraghty, P.J., Mulé, J.J.: Comparative analysis of murine dendritic cells derived from spleen and bone marrow. Proc. Amer. Assoc. Cancer Res. 39: 4156a, 1998.
4. Candido, K., Fields, R.C., Fuller, J.A., Mulé, J.J.: Potent antitumor effects of bone marrow-derived dendritic cells in a murine model of breast cancer. FASEB J. 12: A277, 1998.
5. Chen, B.-G., Shi, Y., Smith, J.D., Choi, D., Geiger, J.D., and Mulé, J.J.: The role of TNF-alpha in modulating the quantity of peripheral blood-derived, cytokine-driven human dendritic cells and its role in enhancing the quality of dendritic cell function in presenting soluble antigens to CD4<sup>+</sup> T cells in vitro. Blood 91: 4652-4661, 1998.
6. Fields, R.C., Shimizu, K. and Mulé, J.J.: Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc. Natl. Acad. Sci. USA 95: 9482, 1998.
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#### **C. Funding Obtained or Pending Based on Work Supported by this Award:**

1. U.S. Army Research Office: Augmentation Awards for Science & Engineering Research Training (AASERT): DAAG55-97-1-0239 (Principal Investigator, J.J. Mulé).
2. NCI-NIH 1 R01 CA87019-01: "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer" funding period: 07/01/2000 - 06/30/2004 (Principal Investigator, J.J. Mulé).
3. NCI-NIH 2 P01 CA50327: "Cellular Vaccines for Cancer Therapy: SLC and Dendritic Cells in Antitumor Immunity and Therapy" funding period: pending (Co-Principal Investigators, A.E. Chang and J.J. Mulé)

#### **CONCLUSIONS/SIGNIFICANCE:**

The significance of our research lies in the potential to develop new, innovative vaccine strategies for eventual use in breast cancer patients that employs the chemokine SLC (and interleukin-2) combined with dendritic cells to both recruit/concentrate relevant immune populations at the vaccination site (by secreted SLC) as well as to activate the recruited T cells by potent presentation of tumor-associated antigens (by dendritic cells and interleukin-2). This approach may prove to be a highly effective means of both detecting and augmenting the immune response to poorly-immunogenic breast tumors that ultimately leads to tumor eradication. In addition, preclinical animal studies that have linked breast tumor apoptosis with susceptibility to treatment with dendritic cells have the potential to spawn a new generation of breast cancer clinical trials to evaluate the combination of apoptosis-inducing agents with SLC-producing dendritic cells in patients with accessible disease.

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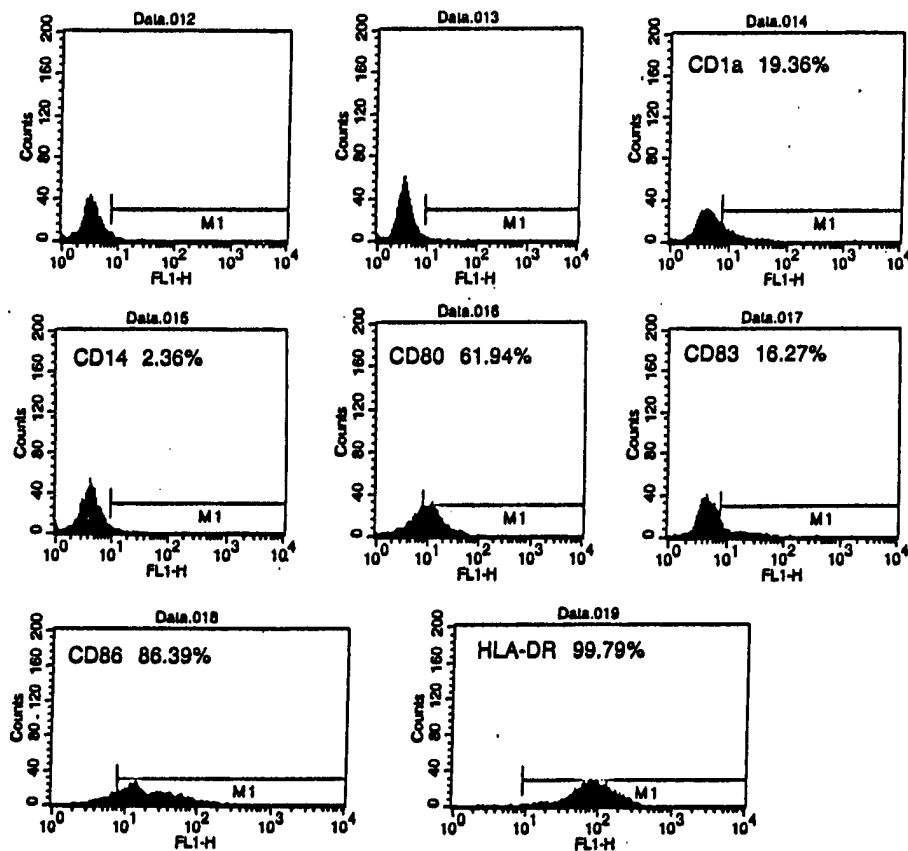
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FIGURE 1

Day 8 ( replated ) DC from P051



Day 8 ( replated ) DC from P051 1ug/ml CD40L treatment

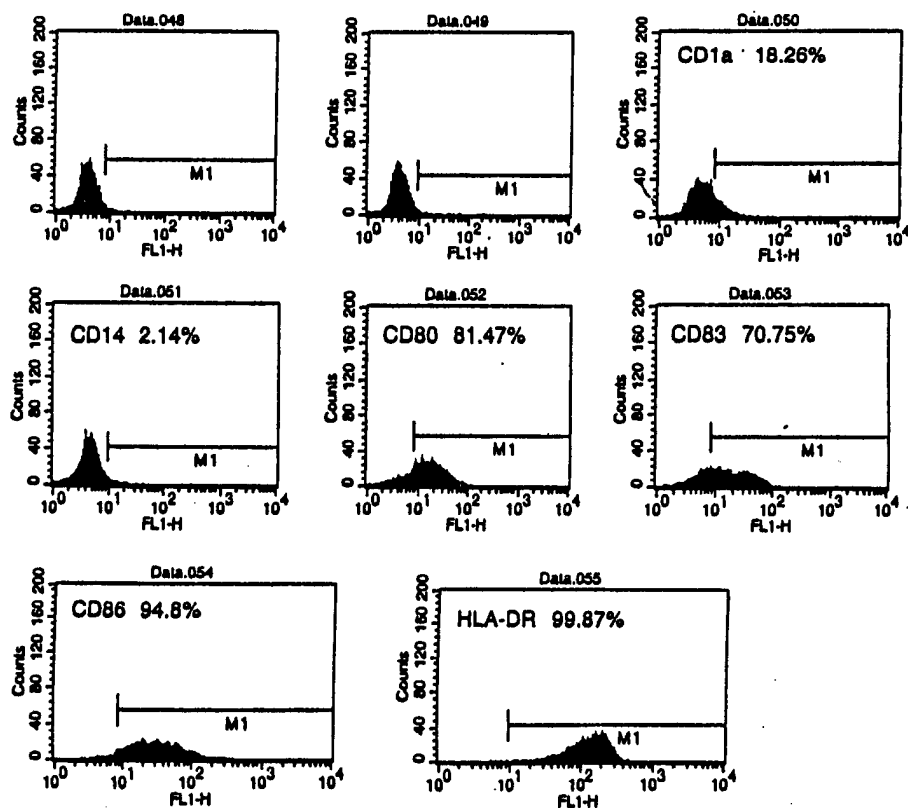
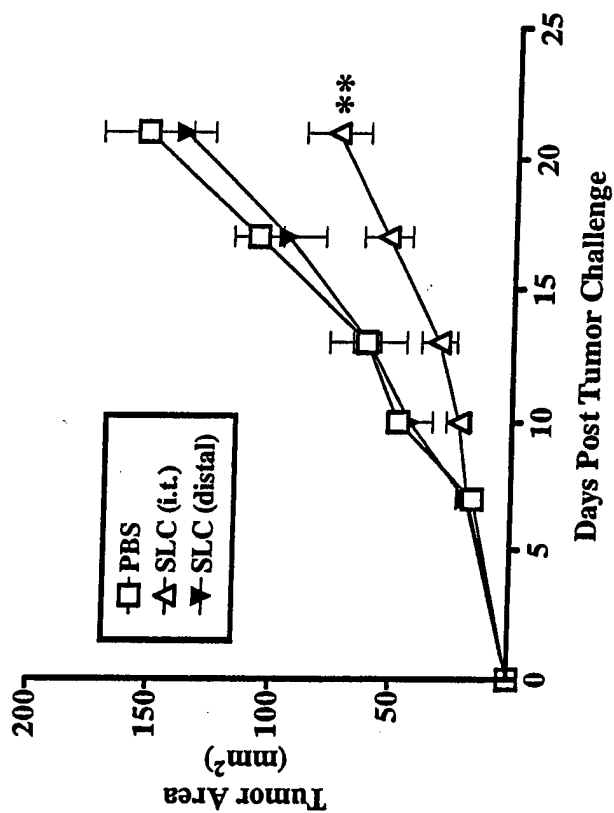


FIGURE 2

B



A

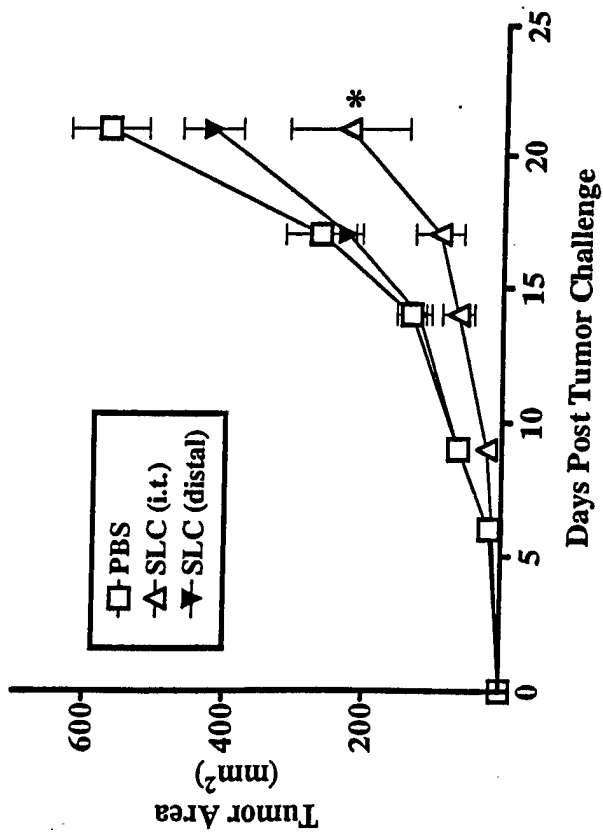
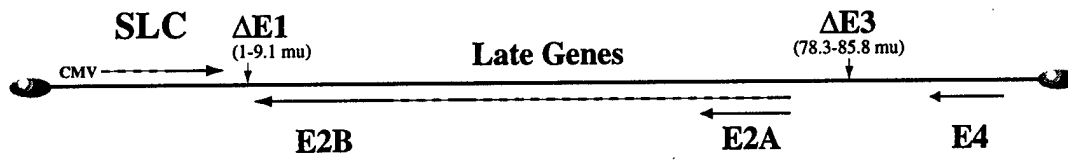
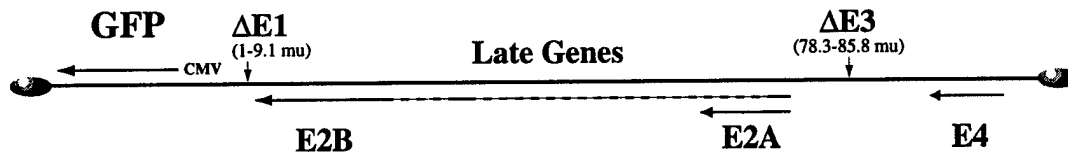


FIGURE 3

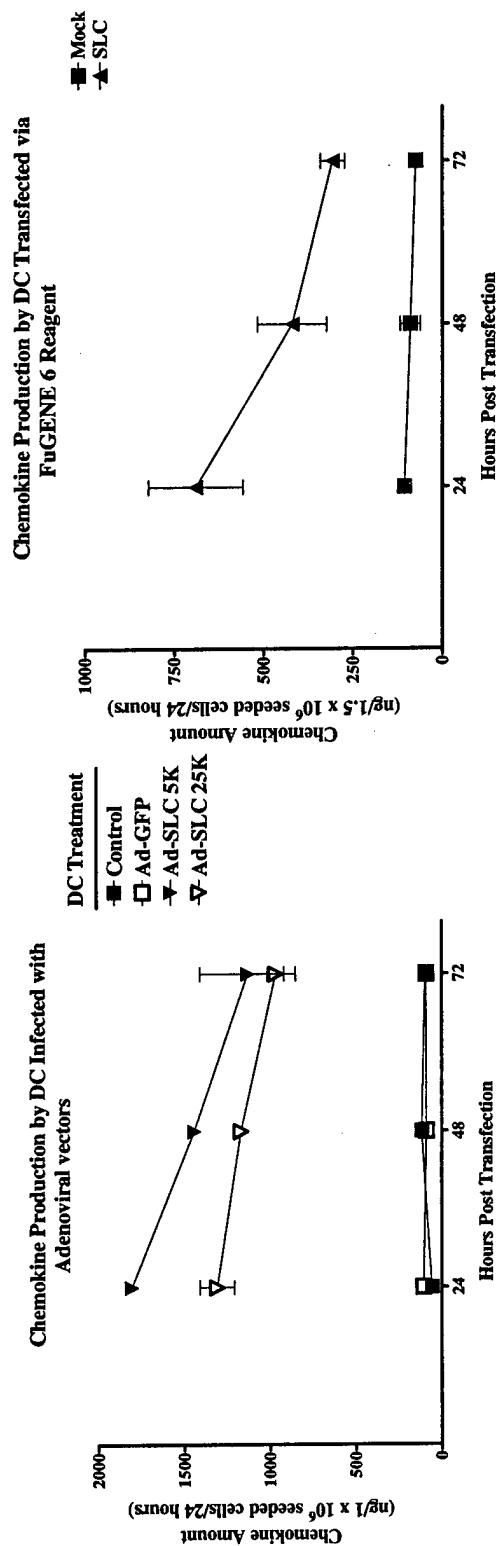
### aD2028#16



### pAdEasy-GFP



# Adenovirus Transfection of DC is Superior to Lipid Mediated Transfection



5K = 5,000 transducing units/DC  
25K = 25,000 transducing /DC

FIGURE 4

# Adenoviral Mediated Transfection of Murine Dendritic Cells

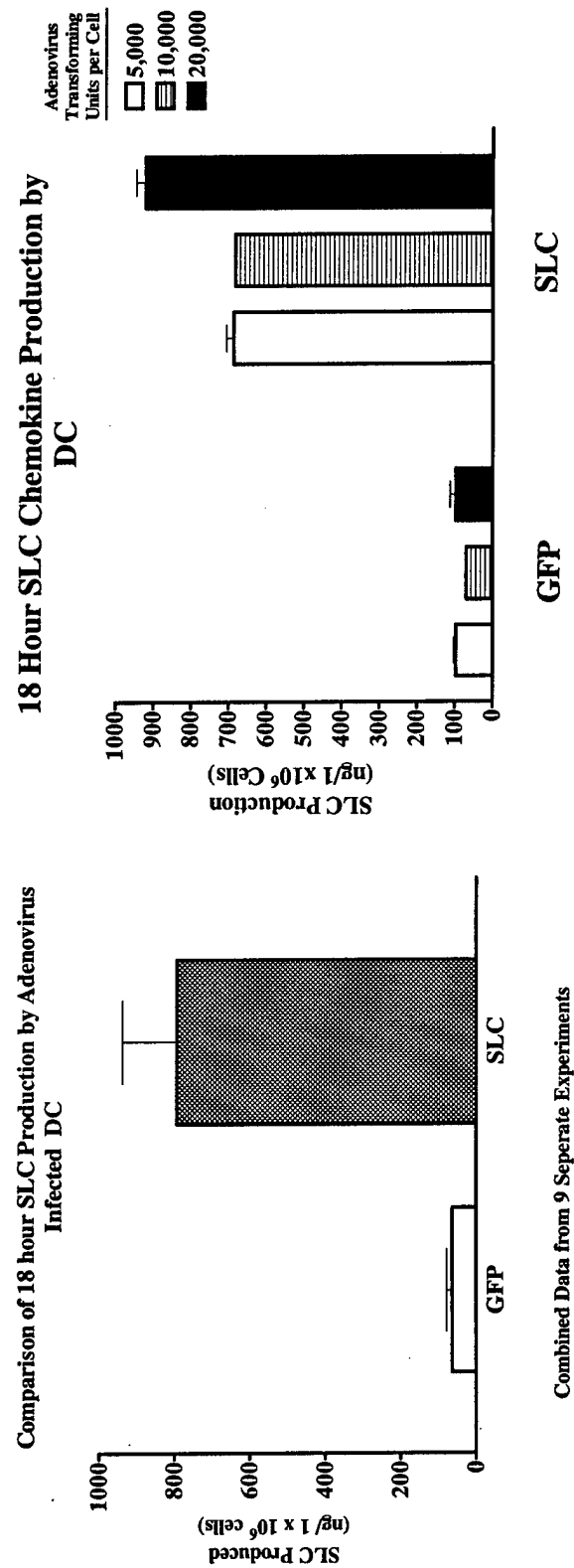


FIGURE 5

FIGURE 6

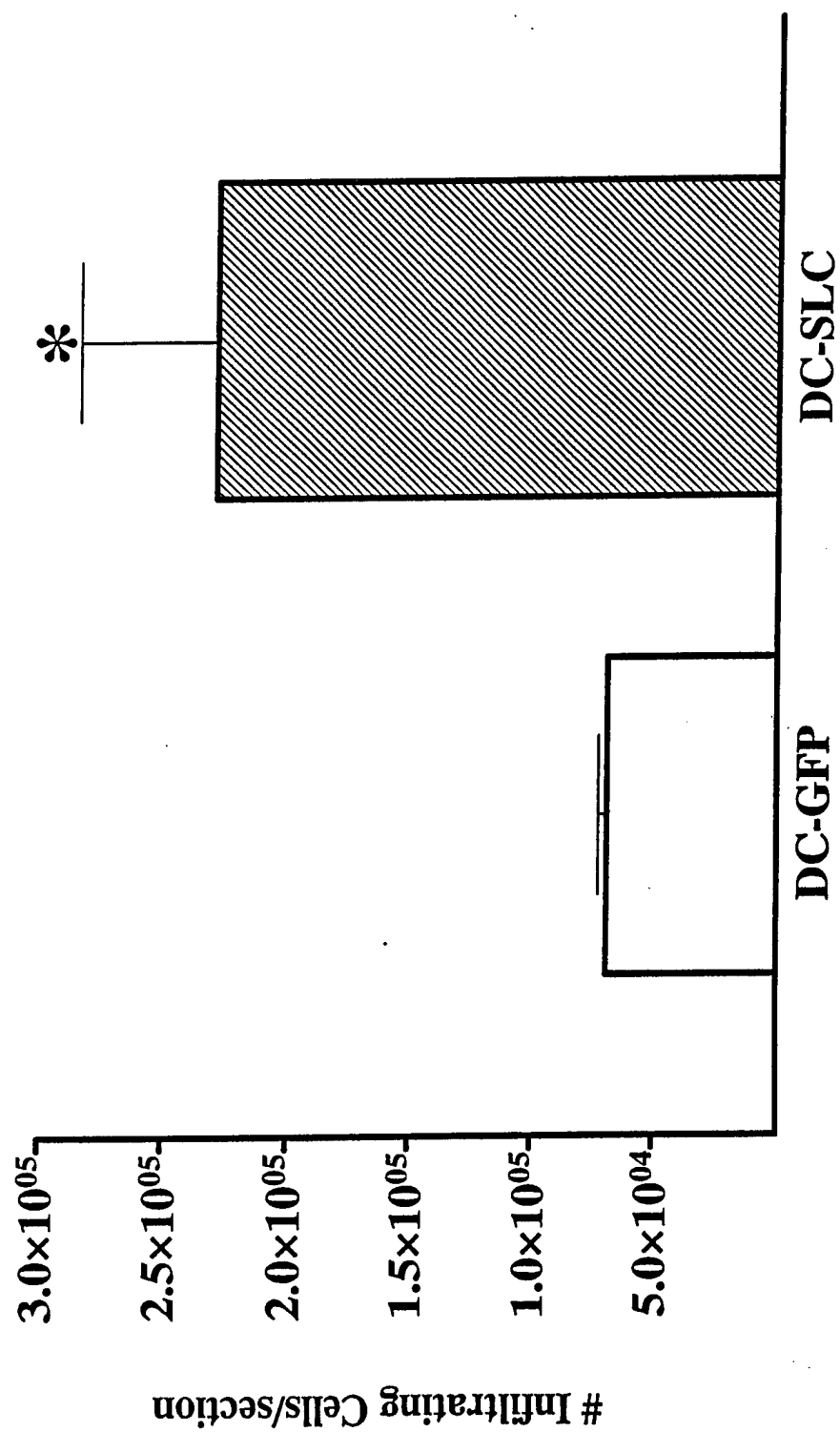
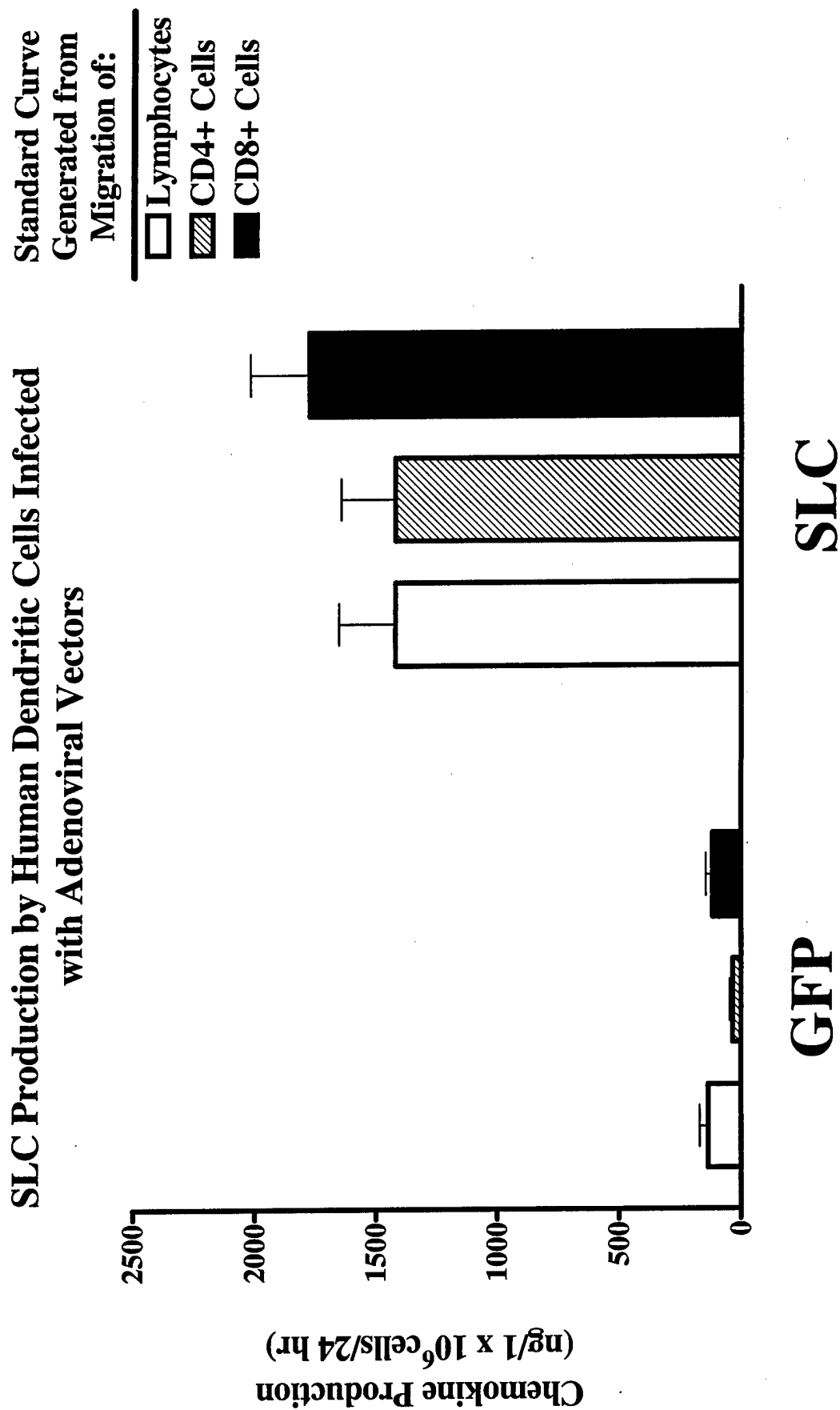


FIGURE 7



# Dendritic cells: at the clinical crossroads

## Commentary

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See related article,  
pages R9-R14

Dendritic cells (DCs) are highly potent antigen-presenting cells of bone marrow origin that can stimulate both primary and secondary T- and B-cell responses (1). First described by Steinman, DCs display a characteristic veiled appearance with multiple extending cellular processes. These cells possess the necessary components for potent antigen-presenting functions, including the production of a variety of important immunostimulatory cytokines and the expression of critical cell-surface molecules. Depending on their level of maturity, DCs express prominent levels of MHC class I and class II molecules, as well as costimulatory molecules such as CD40, CD80, and CD86. Animal studies show them to be responsible primarily for sensitizing naive T cells in their first exposure to antigen. Because of this unique property in inducing immunity, DCs have been termed "nature's adjuvant" (1).

Antigen distribution in the host environment often favors uptake and presentation by DCs rather than macrophages or B cells, and subsequent migration of primed DCs to lymphoid organs enhances targeted presentation of antigens to the immune system. More recently, it has also been shown that murine monocytes residing in subcutaneous tissue can become lymph-borne DCs that localize in draining lymph nodes (2). Once in the lymph nodes, these DCs can present both MHC class I- and class II-restricted antigens and can therefore stimulate both resident CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Whether the migration to the lymph nodes is strictly required for DCs to be competent to stimulate these responses remains less certain. In this regard, Banchereau's group has reported that in human breast carcinomas, immature DCs can reside within the tumor mass itself, whereas the mature ones are located in

peritumoral areas (3). As possible evidence of an ongoing immune response in situ, some peritumoral areas of the specimens showed T cells clustered around the mature DCs, which resembled clusters often reported for secondary lymphoid organs.

The maturation state of DCs appears to be important for their optimal use in immunization strategies, with more mature DCs demonstrating higher production of some key cytokines (e.g., IL-12), increased antigen presentation in vitro and in vivo, and, at least in mice, increased localization to draining lymph nodes and more potent induction of broad T-cell immunity and antitumor activity (1, 4). CD40L, LPS, monocyte-conditioned medium, and TNF $\alpha$  have all

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**T**hese cells possess the necessary components for potent antigen-presenting functions. . .

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been used to promote DC maturation. Human DCs can arise from bone marrow-derived and cord blood-derived CD34<sup>+</sup> hematopoietic cell progenitors and also from PBMCs and CD14<sup>+</sup> blood monocytes. Highly enriched rodent or human DCs can now be produced in great numbers by culturing progenitor cells in the presence of cytokines, notably GM-CSF and IL-4, with or without TNF $\alpha$ .

Virally infected human DCs can elicit potent proliferative and cytolytic T-cell reactivity in vitro (5). In animal models, immunization with antigen-presenting DCs can result in strong protective immunity to viruses (e.g., lymphocytic choriomeningitis virus, LCMV) and to tumors (4, 6, 7). With respect to the latter, tumor

antigen-pulsed DCs can successfully treat established mouse tumors in vivo. Tumor-associated and model antigens, in the form of whole cell lysates, apoptotic cell bodies, peptides, proteins, RNA, and DNA have been used and may initiate tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses (7, 8).

DCs have now reached a watershed — their efficacy in immunization approaches for the protection from and the treatment of human disease is finally being tested. The establishment of human DC cultures from the peripheral blood of patients has facilitated their use as immunotherapeutic agents, most notably in the treatment of infectious diseases and against a variety of human tumors. Initial clinical trials involving DC-based immunization of patients with tumors of hematologic and solid origin are promising: subjects show increased anti-tumor T-cell reactivity and experience partial or complete clinical responses (9–14). However, meaningful comparisons of the immunologic and clinical outcomes of these trials

have been complicated by variability regarding the source of the DCs, their level of maturity, the nature of the antigen used to pretreat them, as well as the dosing regimen and route of administration used. An additional complication centers on the fact that these immunizations have been conducted in advanced cancer patients with various tumor types, at different stages of disease, and with different histories of previous therapy.

The study of Dhodapkar et al. reported in this issue of the *JCI* represents an important step toward optimizing some of these variables (15). These same investigators reported earlier in the *JCI* that, remarkably, a single subcutaneous injection of fewer than  $3 \times 10^6$  mature DCs could rapidly expand



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# Potential of Immunologic Responsiveness to Dendritic Cell-Based Tumor Vaccines by Recombinant Interleukin-2

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## ■ PURPOSE

Dendritic cells (DC) can elicit potent immune responses to tumors through their capacity to efficiently process and present tumor-associated antigens. In a variety of animal tumor models, vaccines based on tumor lysate-pulsed DC (TP-DC) have been shown to effectively immunize against lethal tumor challenges as well as to treat established growing tumors at skin and organ sites. The antitumor effects elicited by TP-DC-based vaccines in vivo have been shown to be mediated by tumor-specific proliferative, cytotoxic, and cytokine-secreting host-derived T cells. Because of the critical involvement of T cells in the antitumor immune response, we have been investigating whether the systemic administration of recombinant interleukin (IL)-2 can enhance the therapeutic efficacy of TP-DC-based tumor vaccines.

## ■ MATERIALS AND METHODS

Immunization with TP-DC plus IL-2 administration was evaluated to determine if this combination could enhance protective immunity toward a weakly immunogenic sarcoma (MCA-207) and a poorly immunogenic subline (D5) of the B16 melanoma and mediate therapeutic rejection of established tumors in C57BL/6 (B6) mouse models.

## ■ RESULTS

We have demonstrated in our murine models that the addition of IL-2 at relatively nontoxic doses can markedly augment the antitumor activity of TP-DC-based tumor vaccine therapies against both a weakly immunogenic sarcoma and a poorly immunogenic melanoma. Animals treated with the combination exhibited significantly greater protection from tumor-cell challenge, significantly greater regression of established tumors, and significantly longer mean survival time than with either TP-DC or IL-2 therapy alone. The mechanism operative in vivo appears to involve the enhancement of immune T-cell function.

## ■ CONCLUSION

These preclinical studies demonstrate the potential of this novel treatment strategy and support the rationale for planned phase I/II clinical trials of TP-DC-based vaccines plus IL-2 in patients with advanced melanoma and colorectal cancer. (*Cancer J Sci Am* 2000;6[suppl 1]:S67-S75)

**Key words:** Cancer vaccines, dendritic cells, immunotherapy, interleukin-2

Previous studies have shown that recombinant interleukin (IL)-2 can enhance the effectiveness of tumor vaccines. Immunization with a combination of irradiated tumor cells and systemic administration of IL-2 has resulted in increased survival of mice upon challenge

with a lethal dose of tumor cells of both an alveolar cell carcinoma<sup>1</sup> and a B16 melanoma.<sup>2</sup> Several studies have also demonstrated that IL-2 administration can augment active-specific immunotherapy with vaccinia virus-modified tumor cell lysates.<sup>3-5</sup> When a vaccinia colon oncolysate was used in combination with exogenously administered IL-2, a reduction in the number of hepatic colon metastases as well as an increase in the survival of mice was observed.<sup>3,4</sup> In another murine system in which the tumor expressed a model "tumor antigen" (i.e.,  $\beta$ -galactosidase), the systemic administration of IL-2 improved the antitumor effectiveness of immunization with the  $\beta$ -galactosidase-expressing recombinant vaccinia virus.<sup>6</sup> Similar studies by others have shown that IL-2 could enhance the therapeutic efficacy of a recombinant vaccinia

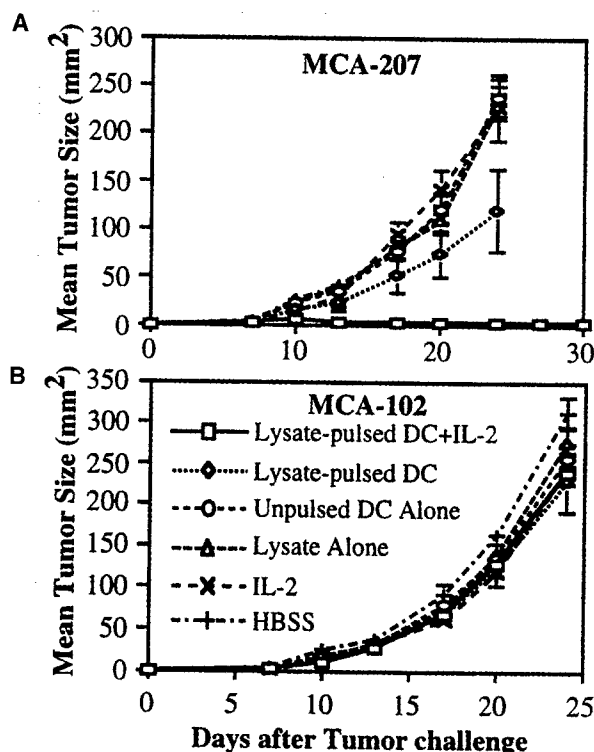
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**Figure 1** Systemic administration of IL-2 can enhance the efficacy of TP-DC to induce specific protective immunity to lethal tumor challenge. Mice were immunized twice SC in the right flank with MCA-207 TP-DC and received intraperitoneal injections of IL-2 after each immunization. Control groups of mice received saline (HBSS), tumor lysate alone, unpulsed DC alone, MCA-207 TP-DC alone, or IL-2 alone. All mice were then re-challenged with a lethal dose of either (A) viable MCA-207 or (B) unrelated MCA-102 tumor cells by SC injection in the left flank. Data are reported as average tumor area  $\pm$  standard error of the mean of five or more mice/group.

virus–carcinoembryonic antigen (rV-CEA) vaccine.<sup>7</sup> Of particular importance, splenocytes from mice treated with rV-CEA plus IL-2 showed enhanced antigen-specific proliferation, cytotoxicity, and interferon gamma (IFN- $\gamma$ ) secretion. In nontumor systems, investigators have shown IL-2 to be a potent activator of immune responses that improve the protective as well as the therapeutic potential of vaccines against pathogens.<sup>8,9</sup>

Our laboratories have been investigating strategies to enhance tumor vaccines based on the use of dendritic cells (DC). Dendritic cells are potent antigen-presenting cells that can elicit primary and boost secondary immune responses to foreign antigens.<sup>10,11</sup> In a variety of settings, these specialized antigen-presenting cells can induce both the generation and proliferation of specific cytotoxic T lymphocytes (CTL) and T helper cells via antigen presentation by major histocompatibility complex (MHC) class I and class II molecules, respectively. Wei<sup>12-14</sup> and others<sup>15-17</sup> have described the induction of MHC class I and class II specific T-cell responses following stimulation with tumor antigen-pulsed DC in vitro and in vivo. Because of these properties, much attention has been directed toward the use of DC in vaccine strategies for the treatment of cancer. In this regard,

DC pulsed with tumor-associated antigen(s) in various forms, including whole cell lysates,<sup>12-14</sup> peptides,<sup>15,16</sup> proteins,<sup>18</sup> RNA,<sup>19</sup> and DNA,<sup>20,21</sup> have been studied for antitumor effects in experimental animals and have demonstrated promising activity. In these models, immunization with tumor antigen(s) presented by DC has effectively primed the cellular immune response and has elicited tumor regression in vivo. Of importance, host-derived CD8+ and, to a lesser extent, CD4+ T cells have been shown to play the central role in the antitumor effects mediated by DC-based vaccines.<sup>14</sup>

The enhancement of DC function by cytokines can occur at two levels: cytokines can directly affect the generation and maturation of DC<sup>22,23</sup> and indirectly affect the elicited T-cell response. Because successful cancer therapy with DC-based vaccines has been shown to be dependent on host immune T cells, we have evaluated the systemic administration of IL-2 for its capacity to enhance tumor lysate-pulsed DC (TP-DC) activity and promote protective immunity. We evaluated whether immunization with TP-DC plus IL-2 administration could enhance protective immunity toward a weakly immunogenic sarcoma cell line (MCA-207) and a poorly immunogenic subline (D5) of the B16 melanoma cell line and mediate therapeutic rejection of established tumors in C57BL/6 (B6) mouse models. Collectively, our results show that IL-2 can enhance the antitumor effects of TP-DC in vivo during both primary immunization and treatment of established tumors.

## ■ MATERIALS AND METHODS

In the MCA-207 sarcoma model, naive B6 mice were immunized twice subcutaneously (SC) at 7-day intervals with  $1 \times 10^6$  bone marrow-derived TP-DC, and IL-2 (20,000 or 40,000 IU) was administered by intraperitoneal (IP) injection twice daily for 5 consecutive days after each immunization. The immunized mice were then challenged with a relatively high dose of viable MCA-207 sarcoma cells. Next, we evaluated the effect of immunization with TP-DC plus IL-2 administration on pulmonary metastases. B6 mice harboring MCA-207 pulmonary metastases were immunized twice with TP-DC either on days 3 and 7 (treatment of 3-day nodules) or on days 7 and 11 (treatment of 7-day nodules) after tumor injection, and IL-2 (20,000 and 40,000 IU, respectively) was administered IP twice daily for 7 consecutive days, starting after the first TP-DC injection. Third, the therapeutic effect of TP-DC plus IL-2 was evaluated in mice bearing well-established SC MCA-207 tumors. These mice received three SC injections of TP-DC, 1 week apart, and IL-2 (20,000 IU) was administered IP twice daily for 5 consecutive days after each immunization.

We have also evaluated whether or not IL-2 could enhance TP-DC-mediated antitumor responses to the D5 subline of the B16 melanoma cell line. Naive B6 mice were immunized three times SC at 7-day intervals with TP-DC, and IL-2 (225,000 IU) was administered IP twice daily for 5 consecutive days after each immuniza-

tion. Immunized mice were then challenged with a lethal dose of viable D5 melanoma cells. In addition, B6 mice harboring D5 lung micrometastases were immunized SC with TP-DC on days 3, 7, and 11 after tumor injection, and IL-2 (450,000 IU) was administered IP twice daily for 3 consecutive days after each immunization.

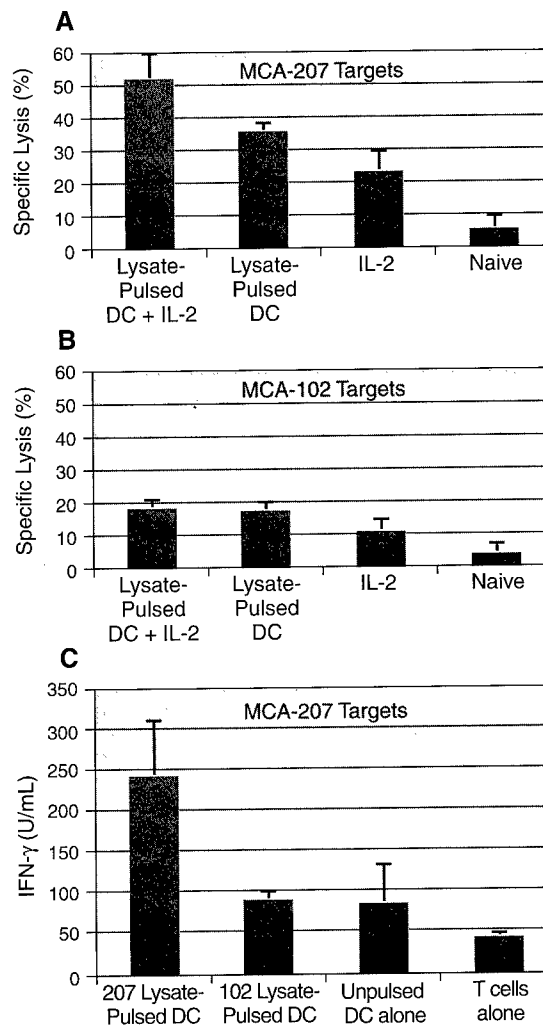
In all our vaccine studies, murine DC were generated by culture of whole bone marrow cells in granulocyte-macrophage colony-stimulating factor (GM-CSF) plus IL-4, as described.<sup>14</sup> Lysates of whole tumor cells were prepared by three rapid freeze-thaw cycles and were coincubated with DC for 18 hours before use as the vaccine preparation.<sup>12-14</sup>

## RESULTS

### MCA-207 Sarcoma Model

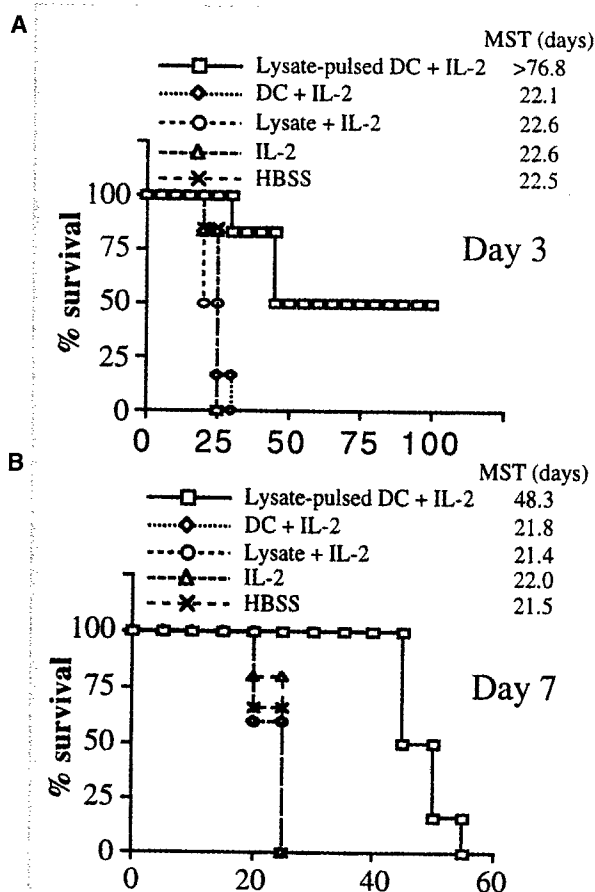
We have recently shown that immunization with MCA-207 TP-DC protects C57BL/6 (B6) mice from a subsequent challenge with viable, syngeneic MCA-207 sarcoma cells.<sup>14</sup> To determine whether IL-2 could enhance immunization by TP-DC in the MCA-207 sarcoma model, B6 mice were subsequently challenged subcutaneously with a threefold higher tumor cell dose than in our previous studies without cytokine administration. As shown in Figure 1A, although immunization with TP-DC alone resulted in a significantly slower growth rate than in control groups ( $P < 0.01$ ), all mice developed progressively growing tumors upon challenge with the higher dose of MCA-207 sarcoma cells. In contrast, all mice treated with the combination of TP-DC plus IL-2 (20,000 IU) were protected from tumor growth and remained disease-free. Immunization of mice with MCA-207 TP-DC with or without the systemic administration of IL-2 had no effect on the growth of the unrelated MCA-102 sarcoma upon SC challenge (Fig. 1B), thus demonstrating the specificity of DC-based immunization.

We also examined the level of CTL activity and IFN- $\gamma$  production in the spleens of these treated and control mice. As shown in Figure 2A, CTL obtained from mice treated with the combination of MCA-207 TP-DC plus IL-2 demonstrated a significantly higher level of specific lysis of the MCA-207 tumor target than did groups receiving either treatment alone ( $P < 0.05$ ). In contrast, these CTL demonstrated low-level lytic activity toward the unrelated MCA-102 tumor target (Fig. 2B). Splenic T cells isolated from mice treated with the combination therapy also produced greater amounts of IFN- $\gamma$  (> 200 U/mL) when specifically stimulated in vitro with MCA-207 TP-DC (Fig. 2C). Splenic T cells obtained from mice treated with MCA-207 TP-DC or IL-2 alone or left untreated produced IFN- $\gamma$  at levels ranging from approximately 50 U/mL to undetectable levels (data not shown). Collectively, these findings demonstrated that the systemic administration of IL-2 could enhance the antitumor efficacy of TP-DC immunization in vivo; IL-2 induced a higher level of CTL activity, IFN- $\gamma$  production, and protective immunity to lethal tumor cell challenge.



**Figure 2** Systemic administration of IL-2 can enhance the capacity of MCA-207 TP-DC to induce (A and B) tumor-specific cytotoxic T-lymphocyte and (C) interferon gamma (IFN- $\gamma$ ) production in vivo. Mice were immunized twice weekly following the schedule described in Figure 1. Spleens were harvested 7 days after the final IL-2 or saline (HBSS) injection from mice treated with HBSS, MCA-207 TP-DC plus IL-2, MCA-207 TP-DC alone, or IL-2 alone. Values are the mean  $\pm$  standard error of the mean (SEM) of triplicate wells at a 100:1 effector:target ratio. For measurement of (C) IFN- $\gamma$  production, splenic T cells from the treated mice were stimulated in vitro. Culture supernatants were harvested 48 hours later and evaluated for IFN- $\gamma$  levels by standard enzyme-linked immunosorbent assay (in U/mL; mean  $\pm$  SEM of triplicate samples).

**Treatment of established MCA-207 tumors.** To determine the therapeutic potential of immunization with the combination of TP-DC plus IL-2, we attempted to induce tumor rejection in mice with established tumors. In a 3-day treatment model, B6 mice harboring pulmonary micrometastases were treated with the combination of TP-DC and nontoxic doses of IL-2 (20,000 IU); comparisons were made with control groups of mice receiving either no treatment or treatment with single agents alone. As shown in Table 1, mice treated with TP-DC alone demonstrated approximately a 90% reduction in the number of established 3-day pulmonary metas-



**Figure 3** Treatment with the combination of TP-DC and IL-2 can prolong survival and result in cures of mice bearing established pulmonary metastases. Mice harboring MCA-207 pulmonary metastases were immunized three times on days 3, 7, and 11 (for 3-day treatment model; panel A) or days 7, 10, and 13 (for 7-day treatment model; panel B) after tumor injection. IL-2 was given intraperitoneally twice daily at (A) 20,000 IU/dose or (B) 40,000 IU/dose for 3 consecutive days after each immunization. Control groups of mice received saline (HBSS), tumor lysate plus IL-2, unpulsed DC plus IL-2, or IL-2 alone. Survival was monitored over time after tumor injection, and the mean survival time (MST) in days was determined.

tases compared with the control groups ( $P < 0.01$ ), as previously reported.<sup>14</sup> Nevertheless, the combination of TP-DC and IL-2 resulted in a further reduction in the number of lung nodules. In this regard, 60% of mice treated with the combination had no detectable pulmonary metastases, and the remaining 40% of mice had fewer than 10 nodules ( $P < 0.01$ ). The addition of a similar course of IL-2 injections to unpulsed DC did not significantly alter the very modest antitumor effect of either alone (data not shown).

We also evaluated the therapeutic efficacy of the combination of TP-DC and nontoxic doses of IL-2 (40,000 IU) on grossly visible, 7-day pulmonary macrometastases from the MCA-207 sarcoma. Treatment with TP-DC alone, IL-2 alone, or tumor lysate plus IL-2 could mediate a significant, albeit modest, reduction (approximately 25%) in the number of 7-day pulmonary metastases compared with saline, tumor lysate alone, or

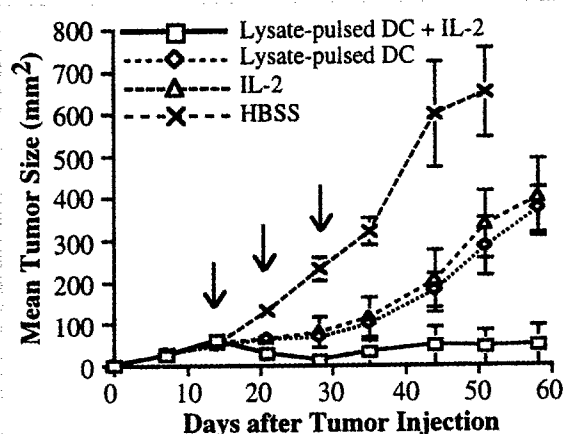
unpulsed DC alone (Table 1). By contrast, the combination of TP-DC plus IL-2 resulted in a demonstrable antitumor effect. All mice treated with the combination had fewer than 21 nodules ( $P < 0.01$ ).

In a separate series of experiments, we sought to determine the therapeutic impact of the combination of TP-DC and IL-2 on disease-free survival of mice harboring 3-day or 7-day pulmonary nodules at the time of treatment. In the 3-day model (Fig. 3A), 50% of mice treated with the combination of TP-DC plus IL-2 survived at least 100 days and had no evidence of tumor when the experiment was terminated (mean survival time [MST] > 77 days;  $P < 0.01$ ). In the 7-day model (Fig. 3B), whereas all control groups of mice survived < 25 days, all mice treated with the combination survived for at least 40 days (MST = 48 days;  $P < 0.01$ ). The effectiveness of the combination of TP-DC and approximately a 10-fold higher dose of IL-2 (i.e., 450,000 IU) was also evaluated in the 7-day model. Although no mice with 7-day pulmonary metastases were rendered completely tumor-free after three cycles of the combination therapy with TP-DC and high-dose IL-2, these treated mice survived significantly longer (MST = 75 d;  $P < 0.01$ ) than did mice receiving either TP-DC (MST = 34.4 d) or high-dose IL-2 (MST = 30.4 d) alone (data not shown). Collectively, these findings indicated that treatment with the combination of TP-DC and IL-2 could mediate antitumor effects on established visceral metastatic disease, leading to increased survival and, in some cases, to cure.

We have also evaluated the capacity of the combination of TP-DC and IL-2 (20,000 IU) to affect the growth of established subcutaneous MCA-207 tumors. MCA-207 sarcoma cells were injected subcutaneously in the flank of B6 mice, and 14 days later, combination therapy was given. Control groups of mice received saline, MCA-207 TP-DC alone, or IL-2 alone. As shown in Figure 4, mice treated with TP-DC alone or IL-2 alone experienced significant reductions in tumor growth compared with the control, unrelated groups; however, no mice were rendered tumor-free. In contrast, mice treated with the combination therapy exhibited a demonstrable antitumor effect, with the majority of tumors undergoing regression. Tumor growth was suppressed by 7 days after the first cycle of combination therapy. Figure 5 demonstrates that 80% of mice that had received the combination of TP-DC and IL-2 survived long-term without evidence of tumor recurrence (MST > 134 d;  $P < 0.01$ ). Thus, these findings extend those obtained in the pulmonary metastases model and further demonstrate that the systemic administration of IL-2 can significantly enhance the antitumor effect of TP-DC against established, SC-growing tumors.

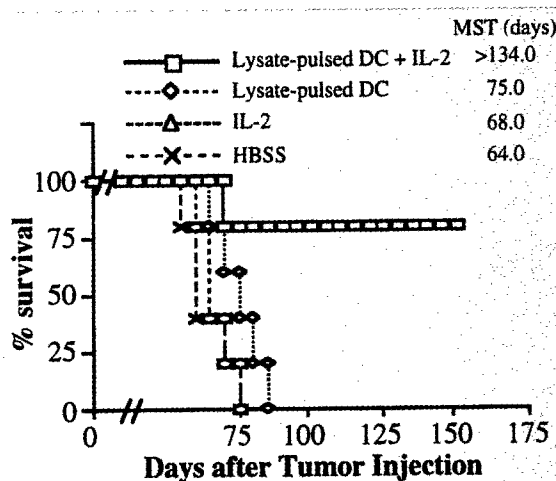
#### B16 Melanoma Model

We next determined whether IL-2 could enhance TP-DC-mediated antitumor responses to the B16-BL6 melanoma, which is of spontaneous origin. A subline of



**Figure 4** Treatment of mice with TP-DC and the systemic administration of IL-2 results in regression of established, 14-day SC tumors. A total of  $2 \times 10^5$  syngeneic MCA-207 sarcoma cells were injected SC in the right flank. Mice bearing 14-day tumors were then treated weekly three times with  $1 \times 10^6$  MCA-207 TP-DC in the left flank. IL-2 was given intraperitoneally twice daily at 20,000 IU/dose for 5 consecutive days after each immunization. Control groups of mice received saline (HBSS), MCA-207 TP-DC alone, or IL-2 alone. The size of the tumors was assessed in a blinded, coded fashion twice weekly and recorded as tumor area (in  $\text{mm}^2$ ).

the B16-BL6 tumor, termed D5, has been characterized previously by our laboratory.<sup>24</sup> The D5 subline expresses few to no detectable surface MHC class I antigens and no detectable surface MHC class II antigens. Previous studies<sup>25,26</sup> have shown that B16 melanoma that expressed little or no MHC class I antigens was not susceptible to treatment with high-dose IL-2 alone. Because we found in our earlier studies that splenocytes from mice immunized with the DC-based vaccine and receiving systemic IL-2 produced significant levels (200–300 IU) of IFN- $\gamma$  (Fig. 2C), we next evaluated whether the ex-



**Figure 5** Combination therapy with TP-DC plus IL-2 can prolong survival and result in cures of mice bearing established, 14-day SC tumors. Mice were treated 14 days after tumor injection as described in Figure 4. Survival was monitored over time after tumor injection, and the mean survival time in days was determined.

pression of MHC class I molecules could be upregulated on the B16 melanoma D5 subline by brief exposure to a low level (i.e., 100 IU) of IFN- $\gamma$ . As shown in Figure 6, MHC class I expression was substantially elevated following exposure of these D5 melanoma cells to IFN- $\gamma$ .

B6 mice were then immunized with D5 melanoma lysate-pulsed DC in the right flank. The mice were subsequently challenged SC with viable D5 melanoma cells in the left flank. High (but relatively nontoxic) doses of IL-2 (225,000 IU) were given by IP injection twice daily for 5 consecutive days after each TP-DC immunization. Among mice immunized with TP-DC alone, 20% were rendered tumor-free (Fig. 7), and this treatment resulted in a significantly slower growth rate of the re-

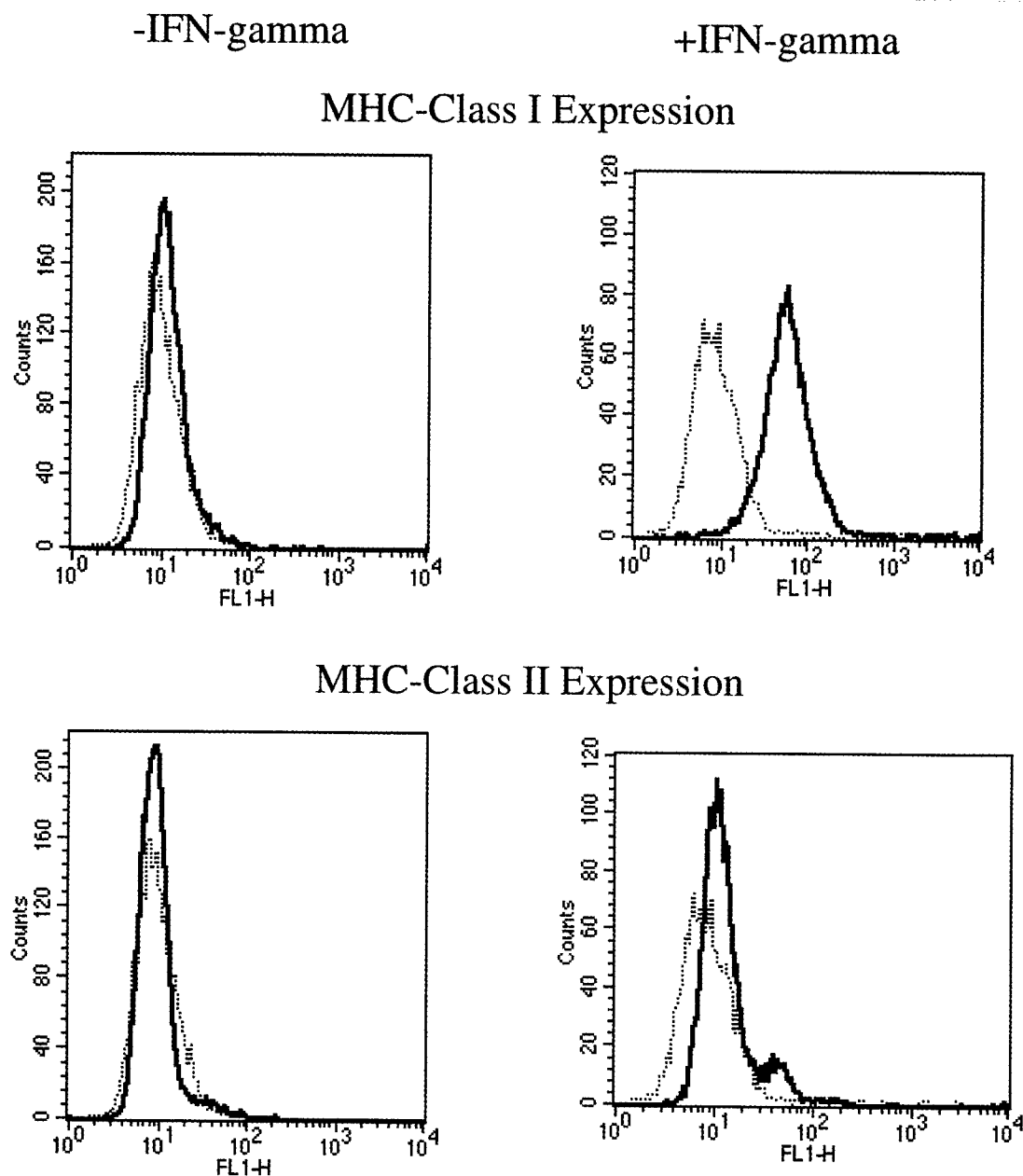
**Table 1. Systemic Administration of IL-2 Can Enhance the Therapeutic Efficacy of TP-DC against Established Pulmonary Metastases**

Pulmonary Metastases	Treatment*	No. of Metastases (range)†	Mean
3-day	HBSS	$\geq 250$	250
	IL-2	143–167	155‡
	Lysate	243–250	249
	Unpulsed DC	217–250	242
	TP-DC	16–42	28‡
	TP-DC + IL-2	0–4	1‡
7-day	HBSS	$\geq 250$	250
	IL-2	141–186	159‡
	Lysate	$\geq 250$	250
	Lysate + IL-2	143–192	163‡
	Unpulsed DC	$\geq 250$	250
	TP-DC	138–184	155‡
	TP-DC + IL-2	4–21	10‡

\*Number of MCA-207 tumor cells injected IV was  $1.5 \times 10^5$ . A total of  $1 \times 10^6$  MCA-207 TP-DC were injected twice on days 3 and 7 or days 7 and 11 after tumor injection. IL-2 was administered IP twice daily at 20,000 IU/dose on days 3–9 or 40,000 IU/dose on days 7–13.

†Mice were euthanized on day 14 (3-day model) or day 15 (7-day model).

‡Mann-Whitney U test;  $P < 0.01$  (compared with group receiving HBSS).



**Figure 6** Exposure of D5 melanoma cells to IFN- $\gamma$  results in upregulation of their expression of MHC class I but not MHC class II molecules. D5 melanoma cells were cultured in the presence of 100 IU of recombinant IFN- $\gamma$  for 12 h. The tumor cells were then harvested and stained with specific monoclonal antibodies (mAbs) to MHC class I (upper panels) or to MHC class II (lower panels) molecules. The stained melanoma cells were then analyzed by flow cytometry. In the histograms, the darker lines represent staining with the appropriate mAb, and the lighter lines represent the control isotype-matched irrelevant mAb (i.e., background staining).

maintaining tumors than that in control groups (not shown). In contrast, 60% of mice immunized with the combination of TP-DC plus high-dose IL-2 were protected from tumor growth upon challenge and remained disease-free (Fig. 7).

**Treatment of established D5 tumors.** We also evaluated the therapeutic efficacy of the combination of TP-DC and high (but relatively nontoxic) doses of IL-2 (450,000 IU) in a 3-day lung tumor treatment model. B6 mice harboring pulmonary micrometastases of the D5 melanoma were treated with the combination of

TP-DC and IL-2. As shown in Table 2, mice receiving TP-DC alone demonstrated an approximate 60% reduction in the number of established 3-day pulmonary metastases compared with the control groups ( $P < 0.01$ ). In contrast, treatment with the combination of TP-DC and high-dose IL-2 resulted in a > 99% reduction in the number of melanoma nodules; 60% of these mice had no detectable pulmonary metastases, and the remaining 40% had fewer than five nodules ( $P < 0.01$ ). Collectively, these findings indicate that the systemic administration of IL-2 can also augment the efficacy of TP-DC–

**Table 2. Systemic Administration of High-Dose IL-2 Can Enhance the Therapeutic Efficacy of TP-DC against Established 3-Day D5 Pulmonary Metastases**

HBSS	Treatment*				Metastases (No.) <sup>†</sup>	Mean $\pm$ SEM	P Value <sup>‡</sup>
	IL-2 (IU/dose)	Lysate Alone	Unpulsed DC Alone	TP-DC			
	450,000						
+	—	—	—	—	250, 250, 250, 250	250	—
—	+	—	—	—	224, 231, 236, 249, 250	238 $\pm$ 5	NS
—	+	+	—	—	160, 241, 241, 250, 250	229 $\pm$ 17	NS
—	+	—	+	—	197, 238, 250, 250, 250	237 $\pm$ 10	NS
—	—	—	—	+	62, 94, 124, 128, 102	102 $\pm$ 15	< 0.01
—	+	—	—	+	0, 0, 0, 2, 3	1 $\pm$ 1	< 0.01

\*Number of D5 tumor cells injected IV was  $1 \times 10^5$ . A total of  $1 \times 10^6$  D5 TP-DC were injected 2 times daily on days 3, 7, and 11 after tumor injection. IL-2 was administered IP twice daily at 450,000 IU/dose for 3 consecutive days after each immunization.

<sup>†</sup>Mice were euthanized at day 14.

<sup>‡</sup>Mann-Whitney U test;  $P < 0.01$  (compared with group receiving HBSS).

based vaccines to induce antitumor activity against the poorly immunogenic D5 melanoma.

## CONCLUSION

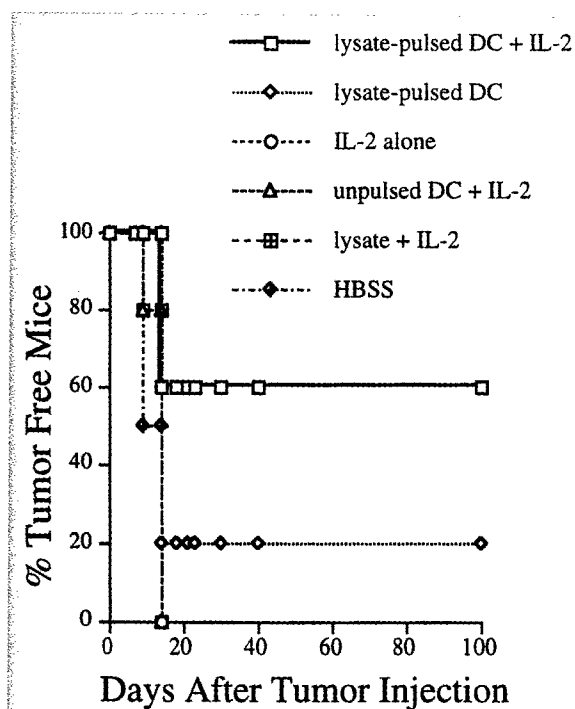
In the current studies, we have demonstrated that the systemic administration of recombinant IL-2 could augment the in vivo antitumor effects of TP-DC injections. We first reported that whole tumor lysates could serve effectively as a source of tumor-associated antigen(s) to elicit specific T-cell responses in vitro when processed and presented by DC.<sup>12,13</sup> In vivo, SC injections of TP-DC from the spleen or bone marrow resulted in effective immune priming of naive mice to reject subsequent challenge with a low dose of viable cells from a syngeneic melanoma,<sup>27</sup> sarcoma,<sup>14</sup> and breast carcinoma.<sup>14</sup> Moreover, immunization of mice with TP-DC could mediate regression of established 3-day pulmonary micrometastases from both a sarcoma and a breast carcinoma.<sup>14</sup> Recently, in a pilot study, human DC pulsed with autologous melanoma lysate could induce antigen-specific immunity during DC vaccination of patients and result in objective clinical responses with the apparent absence of physical signs of autoimmunity.<sup>28</sup> In the murine models, successful immune priming and tumor regression were dependent on the participation of host-derived T cells.<sup>14</sup> Because of this necessity for T cells, it appeared reasonable to hypothesize, based on our earlier work,<sup>29,30</sup> that the systemic administration of IL-2 may augment the antitumor effects of TP-DC-based vaccine approaches.

We and others have shown previously that the systemic administration of recombinant IL-2 alone could mediate profound antitumor effects in mice when ad-

ministered at high doses.<sup>29,30</sup> Similar results were obtained in certain advanced cancer patients as well.<sup>31</sup> However, dose-limiting toxicities of high-dose IL-2 administration resulted in both animals and humans, and have hampered its wider use as a single agent in cancer therapy.<sup>31-33</sup> Toxicity of IL-2 is manifested by a vascular leak syndrome during and following the elicitation of lymphoid cell proliferation and activation of lymphokine-activated killer cell activity in situ.<sup>32</sup> Given this limitation, attempts have been made to combine IL-2 at lower doses with other cytokines, chemotherapeutic agents, and radiation.<sup>34</sup> More recently, IL-2 has been shown to enhance the therapeutic efficacy of a peptide-based vaccine approach in patients with advanced melanoma.<sup>35</sup>

In our current studies, the systemic administration of IL-2 at doses lower than those required for the successful treatment of established tumors in mice when used as single-agent therapy<sup>28,29</sup> resulted in regression of established tumors when combined with TP-DC immunization in a MCA-207 sarcoma model. Moreover, in a poorly immunogenic D5 melanoma model, although mice bearing 3-day pulmonary metastases treated with high (but relatively nontoxic) doses of IL-2 alone experienced no reduction in the number of pulmonary melanoma nodules, the combination of IL-2 plus TP-DC resulted in a significant reduction in metastatic disease. Confirmatory studies have recently been conducted using apoptotic tumor cells as the source of tumor antigen(s).<sup>36,37</sup> In these experiments in the rat, the efficacy of low-dose IL-2 in combination with vaccines, based on either apoptotic tumor cell bodies alone or apoptotic tumor cell bodies phagocytosed by antigen presenting cells,





**Figure 7** Immunization of mice with TP-DC plus the systemic administration of IL-2 can elicit effective protection against D5 melanoma cells. Mice were immunized 3 times SC at 7-day intervals in the right flank with  $1 \times 10^6$  D5 TP-DC and received intraperitoneal injections of IL-2 at 225,000 IU/ dose for 5 consecutive days after each immunization. Control groups of mice received saline (HBSS), tumor lysate plus IL-2, unpulsed DC plus IL-2, D5 TP-DC alone, or IL-2 alone. Seven days after the final injection of IL-2 (or HBSS), all mice were rechallenged with a lethal dose of  $5 \times 10^4$  D5 viable tumor cells by SC injection in the left flank and were followed for survival.

was demonstrated against a poorly immunogenic glioma. The combination treatments resulted in complete tumor regression and induced specific immune protection.

Our further studies demonstrated that the combination of IL-2 and TP-DC elicited greater CTL activity against relevant target cells in the spleens of immunized mice. We also observed that splenic T cells obtained from mice treated with the combination therapy produced a greater amount of IFN- $\gamma$  compared with single-agent therapy following specific stimulation in vitro with MCA-207 TP-DC. Moreover, the amount of IFN- $\gamma$  produced by these splenocytes was shown to upregulate the expression of MHC class I molecules on the poorly immunogenic B16 melanoma subline D5. These findings are of particular interest because both cytolytic and noncytolytic, tumor-specific, tumor-infiltrating lymphocytes were shown previously to mediate their antitumor therapeutic effect upon adoptive transfer, in part, by specific secretion of IFN- $\gamma$ .<sup>38</sup>

Taken together, these studies have shown that the combination of IL-2 and TP-DC may provide promising new applications of IL-2 in the treatment of cancer. In this respect, we are planning to initiate phase I/II clinical trials in advanced melanoma and colorectal cancer to investigate the safety and potential antitumor activity

of the combination of autologous TP-DC plus low-dose and high-dose IL-2.

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# The Future of Interleukin-2: Enhancing Therapeutic Anticancer Vaccines

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## ■ PURPOSE

The purpose of our efforts is to trigger the immune destruction of established cancer. Interleukin (IL)-2 can mediate the regression of tumors in patients with melanoma and renal cell carcinoma. In animal models, the antitumor effects of IL-2 are mediated by T lymphocytes. Stimulation with specific antigen can enhance the ability of T cells to respond to IL-2 by triggering the rapid upregulation of the high-affinity IL-2 receptor. We are seeking to design recombinant and synthetic vaccines capable of preferentially priming T cells with specificity for tumor cells.

## ■ METHODS

The antitumor activity of experimental vaccines is being studied preclinically using recently developed murine models that employ the mouse homologues of human tumor-associated antigens. Once the most effective experimental vaccines are optimized in experimental animals, clinical trials can be conducted. Vaccines are being evaluated for their ability to mediate the regression of established tumors, and a variety of immunologic correlates are being measured.

## ■ RESULTS

In animal models, vaccines based on molecularly defined tumor-associated antigens expressed in viral vectors or delivered as "naked" DNA stimulate the expansion of CD4+ and CD8+ tumor-specific T lymphocytes. Co-administration of IL-2 with these vaccines dramatically

enhances their ability to mediate the regression of established cancer. In the clinic, treatment of melanoma patients with peptide vaccine and IL-2 resulted in objective responses in approximately 40% of patients, a response rate more than twice that typically achieved with IL-2 alone. Paradoxically, tumor-specific CD8+ T-cell levels were not increased in these patients.

## ■ CONCLUSION

The addition of recombinant and synthetic cancer vaccines to a regimen of IL-2 can result in improved antitumor responses in both animal models and melanoma patients. Vaccine-primed, tumor-specific T cells may preferentially proliferate upon administration of IL-2. The apparent lack of increase in CD8+ T-cell numbers in this setting suggests that the vaccine-primed T cells functionally disappear after a transient period of activation. Preventing the disappearance of activated T cells upon IL-2 administration—for example, by blocking proapoptotic signals—may enhance the therapeutic effectiveness of anticancer vaccines. (*Cancer J Sci Am* 2000;6[suppl 1]:S76-S80)

**Key words:** Adjuvant, cancer vaccines, gp100, interleukin-2, melanoma, T lymphocytes

Interleukin (IL)-2 induces durable complete responses in patients with metastatic melanoma and renal cell carcinoma, yet in only about 20% of patients treated with IL-2 will an objective tumor regression be observed, and only about one third of responding patients (approximately 7% of treated patients) will have a complete re-

sponse.<sup>1-4</sup> Although the mechanism of action of IL-2 therapy in humans is not known with certainty, in mouse models therapeutic effects are largely mediated by T lymphocytes.<sup>5</sup> Administration of IL-2 is thought to cause the widespread expansion of T cells, some of which appear to be specific for antigens presented on the surfaces of tumor cells, and it is presumably these T cells that mediate tumor regression. In fact, IL-2 therapy combined with tumor-specific T lymphocytes expanded in vitro doubled the response rate in melanoma patients (34%) compared with response rates that have been achieved with high-dose IL-2 therapy alone (17%).<sup>3,6</sup> This suggests that an important denominator for the success of IL-2 therapy is the frequency of tumor-specific T cells capable of responding to IL-2. If one could increase the frequency of

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## Review

# Gene-Modified Dendritic Cells for Use in Tumor Vaccines

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### ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells capable of priming activation of naive T cells. Because of their immunostimulatory capacity, immunization with DCs presenting tumor antigens has been proposed as a treatment regimen for cancer. The results from translational research studies and early clinical trials point to the need for improvement of DC-based tumor vaccines before they become a more broadly applicable treatment modality. In this regard, studies suggest that genetic modification of DCs to express tumor antigens and/or immunomodulatory proteins may improve their capacity to promote an antitumor response. Because the DC phenotype is relatively unstable, nonperturbing methods of gene transfer must be employed that do not compromise viability or immunostimulatory capacity. DCs expressing transgenes encoding tumor antigens have been shown to be more potent primers of antitumor immunity both *in vitro* and in animal models of disease; in some measures of immune priming, gene-modified DCs exceeded their soluble antigen-pulsed counterparts. Cytokine gene modification of DCs has improved their capacity to prime tumor antigen-specific T cell responses and promote antitumor immunity *in vivo*. Here, we review the current status of gene-modified DCs in both human and murine studies. Although successful results have been obtained to date in experimental systems, we discuss potential problems that have already arisen and may yet be encountered before gene-modified DCs are more widely applicable for use in human clinical trials.

### OVERVIEW SUMMARY

This review discusses the potential benefits and limitations of genetically modified dendritic cells (DCs) for use in therapeutic antitumor vaccines. We discuss the various systems employed for gene transfer to DCs, including the growing consensus that viral vectors represent the most efficient means of transduction. We also describe data supporting the use of DCs modified to express genes encoding tumor antigens and immunomodulatory proteins, such as cytokines, to promote antitumor immunity. Successful pre-clinical results are described, in which genetically modified DCs are used in *in vitro* immunologic studies with human cells *in vitro* as well as animal tumor models *in vivo*. We point out the benefits and disadvantages of using gene-modified DCs and express our viewpoint on what types of gene-modified DCs may be considered candidates likely to reach the clinic.

### INTRODUCTION

SINCE THEIR DISCOVERY more than 25 years ago, dendritic cells (DCs) have emerged as the most potent member of the class of antigen-presenting cells (APCs) (Banchereau and Steinman, 1998). Because of their potent capacity to stimulate T lymphocytes, particularly naive T cells, DCs have been proposed as the basis for vaccines designed for the treatment of cancer (Cohen *et al.*, 1994; Schuler and Steinman, 1997). Coupled with our understanding that tumors express antigens (TAA, tumor-associated antigen) capable of being recognized by the immune system, DC-based tumor vaccines have been translated from the laboratory to the clinic (Timmerman and Levy, 1999). The results from early phase I clinical trials support the idea that DCs presenting TAA can initiate an antitumor immune response in certain patients and, in some cases, can lead to partial or complete regression of tumors (Hsu *et al.*, 1996; Nestle *et al.*, 1998). Although these early results are encouraging, they also

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point to the exigency for modification of DC-based vaccines before they become a commonly used modality for the treatment of cancer. Gene therapy in the setting of DC-based vaccines represents one such modification. The purpose of this review is to bring together data on gene techniques and translational research using gene-modified DCs. By drawing parallels from animal models and *in vitro* assays utilizing human cells, we hope to highlight the promise entailed in creating the next generation of vaccines based on gene-modified DCs.

### DC generation and morphology

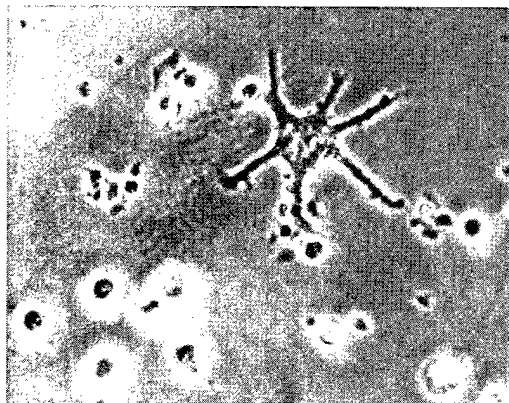
Because DCs can be derived from multiple cell types using a number of different tissue culture conditions, a brief primer on DC generation and phenotype is necessary before we can begin to address issues of gene transfer. DCs are distinguished by their dendritic, veiled morphology (Fig. 1) and high expression levels of both MHC class I and class II as well as costimulatory and adhesion molecules involved in T cell activation such as CD80/CD86 and ICAM-1 (Hart, 1997; Banchereau and Steinman, 1998). DCs reside in the spleen, lymph nodes, and circulation but can arise from cells such as epidermal Langerhans cells or monocytes, which react to stimuli such as inflammatory cytokines.

An important step in the application of DCs in therapeutic settings was the ability to obtain them in large numbers in highly purified form from donors. In both rodents and humans, DCs can be generated from bone marrow or peripheral blood cells; in the case of peripheral blood both circulating CD34<sup>+</sup> hematopoietic progenitor cells and CD14<sup>+</sup> adherent monocytes were found to give rise to DCs under defined tissue culture conditions. On culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4), the resulting cells display the veiled morphology characteristic of DCs and express T cell stimulatory molecules such as MHC I/II, CD80/86, and ICAM-1 (Bender *et al.*, 1996; Romani *et al.*, 1996; Talmor *et al.*, 1998). In humans, these cells represent "immature DCs," defined in part by their high antigen uptake. Further culture of these cells in monocyte conditioned medium

(MCM) or with the addition of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), lipopolysaccharide, type I interferons (IFNs), or IL-1 $\beta$  can increase the expression of T cell stimulatory receptors and induce expression of DC maturation markers such as CD83 (Bender *et al.*, 1996; Luft *et al.*, 1998). These "mature DCs" are more potent T cell stimulators than immature DCs but have reduced phagocytic and endocytic activities (Banchereau and Steinman, 1998). Mature DCs are thought to be better for use in immunization regimens than their immature counterparts because of their increased immunostimulatory capacity, stability of their phenotype, and increased trafficking to lymphoid organs. In the mouse the distinction between immature and mature DCs is not clear-cut due, in part, to lack of a selectable marker for maturation. However, murine bone marrow-derived DCs (BMDCs) cultured in GM-CSF plus IL-4 are functionally more mature (as measured by phagocytic/endocytic activities and T-cell stimulatory capacity) than DCs derived from GM-CSF cultures (Talmor *et al.*, 1998; Labeur *et al.*, 1999). The addition of CD40L or TRANCE, both ligands for TNF receptor family members, has also been shown to promote the maturation and survival of DCs (Wong *et al.*, 1997; Kuniyoshi *et al.*, 1999). Taken together, it appears that multiple modes of DC maturation are capable of resulting in a cell with high immunostimulatory capacity. Whether these different sources of DC progenitors and different protocols for DC generation result in similarly effective antigen-presenting cells is a matter still open to debate but should be kept in mind by the reader of both clinical and translational research reports.

### GENETIC MODIFICATION OF DENDRITIC CELLS

Broadly defined, the target genes transferred into DCs fall into two categories: TAA and immunomodulatory proteins such as cytokines. In the case of DCs expressing TAA, gene constructs encoding whole proteins and cytotoxic T lymphocyte (CTL)-specific epitopes have been utilized. There are a number of potential advantages to using TAA gene-modified DCs. DCs expressing TAA via a transgene should, in theory, present antigen for a longer period than peptide or tumor lysate-pulsed DCs, in which the duration of expression is restricted by the half-life of the peptide-MHC complex created during antigen pulsing. Plasmid DNA is relatively stable and easy to generate, making it a more suitable source of antigen than whole tumor RNA and eliminating the possible transformation of DCs by oncogenes or tumor suppressor genes encoded in tumor DNA. However, TAA gene-transfected DCs present only the antigen encoded by the transgene, resulting in an antigenic restriction not present when tumor lysate or RNA/DNA is used as the antigen source. Because DCs are potent APCs, use of viral gene vectors could result in priming of antiviral immunity, particularly CTLs, resulting in elimination of transfected DCs during subsequent rounds of immunization. Furthermore, the relative instability of the DC phenotype necessitates a method of transfection that does not compromise viability or negatively affect DC functions (Fields *et al.*, 1998). The challenge to the broader use of gene-modified DCs in vaccine applications then is twofold: determine the most efficient means of transfecting DCs



**FIG. 1.** Photomicrograph of a murine dendritic cell. Typical morphology shows a stellate appearance with multiple cell processes. The DC was isolated from spleen as described in Fields *et al.* (1998).

and the optimal target genes to be transfected. In this regard, DCs transfected with genes encoding cytokines or T cell co-stimulatory molecules represent APCs that are potentially more immunostimulatory or longer lived than unmodified DCs.

#### *Efficient gene transfer into DCs via viral vectors*

Published reports of gene-modified DCs have used a variety of gene transfer vehicles including cationic lipids, viral vectors, and plasmid-coated gold particles (Table 1). Gene transfer efficiencies, when reported, ranged from 5% efficiency to greater than 95% transgene-expressing cells. The variation in gene transfer efficiencies may be due, in part, to the transfection vector system employed but may also be due to modifications in gene transfer protocols. Several laboratories have attempted to determine which gene transfer system(s) are optimal for transfecting DCs efficiently. The growing consensus is that viral vectors represent a more efficient and productive means compared with physical or chemical methods. While the majority of published reports detailing virus-mediated transduction efficiency and its effect on DC phenotype have utilized adenovirus, DCs or their progenitors have been transduced with similar efficiencies in studies using retrovirus (Aicher *et al.*, 1997), poxvirus (Brown *et al.*, 1999), and herpesvirus (Coffin *et al.*, 1998).

In direct comparisons of adenoviral vectors with physical methods of transfection such as liposomes, electroporation and CaPO<sub>4</sub>, viral vectors have resulted in consistently higher levels of gene transfer efficiency and expression and transfection efficiencies (90–100 versus 5–10%) (Arthur *et al.*, 1997; Dietz and Vuk-Pavlovic, 1998; Zhong *et al.*, 1999). In fact, most physical methods of transfection were toxic to DCs, resulting in loss of phenotype and substantial cell death (Arthur *et al.*,

1997). Even under optimal conditions when electroporation resulted in 15% transfection efficiency, viability of the DC was less than 60% (Van Tendeloo *et al.*, 1998), and transfection via electroporation occurred in CD34<sup>+</sup> HSC-derived but not in monocyte-derived DCs. However, it should be noted that substantial protein production after gene gun transfection has been achieved, although no determination of transfection efficiency was determined (Tuting *et al.*, 1998). Adenovirus titers of up to 1000–10,000 multiplicities of infection (MOIs) could be well tolerated by DCs as opposed to the M202 and M207 human melanoma cell lines, which demonstrated 40–100% cell death at the same titers (Arthur *et al.*, 1997). Generally, no alterations in DC phenotype after adenoviral infection have been noted (Arthur *et al.*, 1997; Zhong *et al.*, 1999), although there are some reports of increases in T cell stimulatory molecules, such as MHC class I, CD80, and CD86, suggesting that adenovirus may initiate DC maturation (Kaplan *et al.*, 1999; Rea *et al.*, 1999). Transgene expression in adenovirus-infected human DCs could persist for greater than 1 week (Arthur *et al.*, 1997) as opposed to gene gun-mediated transfection, which resulted in only transient expression lasting less than 72 hr (Tuting *et al.*, 1997). When transgene expression from viral vectors was lost rapidly, addition of DC survival factors such as TRANCE could prolong reporter gene expression (although it is not known whether this effect is due to maintenance of the transgene or prolonged survival of the DCs) (Zhong *et al.*, 1999). Adenovirus-transfected DCs were either as effective (Arthur *et al.*, 1997; Zhong *et al.*, 1999) stimulators of the mixed lymphocyte reaction (MLR) or more effective (Kaplan *et al.*, 1999; Rea *et al.*, 1999) than uninfected control DCs; increased stimulatory capacity accompanied increased expression of T cell stimulatory molecules. It appears that adenovirus has been equally capable of mediating gene transfer into human or mouse

TABLE 1. GENE TRANSFER TECHNIQUES EMPLOYED IN DENDRITIC CELL MODIFICATION

<i>Transfection vector</i>	<i>DC source</i>	<i>Comments</i>	<i>References</i>
<b>Virus</b>			
Adenovirus	Human PBMCs	>90% transduction efficiency (MOI >100)	Arthur <i>et al.</i> (1997); Zhong <i>et al.</i> (1999)
Poxvirus	Mouse bone marrow	Antiviral immunity does not affect <i>in vivo</i> efficacy	Kim <i>et al.</i> (1997); Brossart <i>et al.</i> (1997); Brown <i>et al.</i> (1999)
Retrovirus	Human CD34 <sup>+</sup> HSCs	Postinfection DC maturation required	Aicher <i>et al.</i> (1997)
Herpesvirus	Mouse bone marrow	Multiple gene transduction achieved	Specht <i>et al.</i> (1997)
CD40-targeted adenovirus	Human PBMCs	CD40 targeting resulted in DC maturation	Coffin <i>et al.</i> (1998)
Liposome-modified adenovirus	Human PBMCs	Efficient transduction at MOI <100	Tillman <i>et al.</i> (1999)
<b>Physical methods</b>			
CaPO <sub>4</sub> , liposomes	Human PBMCs	>10% transfection efficiency	Dietz and Vuk-Pavlovic (1998)
Electroporation	Human PBMCs	15% transfection efficiency, low DC viability	Arthur <i>et al.</i> (1997); Zhong <i>et al.</i> (1999)
Biolistic device	Human PBMCs	Transient expression (<72 hr)	Van Tendeloo <i>et al.</i> (1998)
	Mouse bone marrow	5–10% transfection efficiency	Tuting <i>et al.</i> (1997); Tuting <i>et al.</i> (1998)

*Abbreviations:* PBMCs, Peripheral blood mononuclear cells; HSCs, hematopoietic stem cells; DC, dendritic cells.

DCs (or DC lines), although no comparative studies have yet been reported (Arthur *et al.*, 1997; Brossart *et al.*, 1997; Ribas *et al.*, 1997; Dietz and Vuk-Pavlovic, 1998; Sonderbye *et al.*, 1998; Kaplan *et al.*, 1999; Zhong *et al.*, 1999).

The requirement for high viral titers (MOI > 100) to achieve efficient gene transfer suggests that DCs lack the adenovirus attachment factor Coxsackie-adenovirus receptor (CAR) or the  $\alpha_v$  integrins,  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$ , used by the virus for fusion with its target cell (Wickham *et al.*, 1993; Bergelson *et al.*, 1997, 1998). Indeed, monocyte-derived DCs cultured in GM-CSF and IL-4 are deficient in CAR but do express the  $\alpha_v$  integrin (Rea *et al.*, 1999; Tillman *et al.*, 1999). Instead, DCs may incorporate virus through phagocytic activity. This is consistent with the observation that gene transfer efficiency and expression levels (as measured by green fluorescent protein [GFP] fluorescence) were greater in immature DCs than in DCs induced to mature by lipopolysaccharide (LPS) or MCM (Zhong *et al.*, 1999). This had led some investigators to use adenovirus to enhance uptake of conjugated DNA particles or to augment adenovirus infection with antibodies or liposomes. Successful gene transfer to both mouse and human DCs has resulted using adenovirus to enhance the uptake of DNA bound to chemical linkers such as poly-L-lysine (Mulders *et al.*, 1998), modified receptor proteins such as transferrin (Curiel-Lewandrowski *et al.*, 1999) and modified chemical linkers targeted to DC receptors such as mannosylated polyethylenimine (Diebold *et al.*, 1999a,b). These systems, which used the virus to aid in DNA/conjugate uptake, were purported to be less toxic than using high-titer adenovirus and would result in a reduced induction of antiadenovirus immunity. Other investigators have attempted to modify adenoviral delivery to DCs by additional strategies. In this regard, adenovirus targeted to CD40 or  $\beta_1$  integrins on human DCs by a bispecific antibody led to increased reporter gene (luciferase) activity compared with adenovirus alone, even at relatively low MOI (<100) (Tillman *et al.*, 1999). Besides improving gene transfer efficiency, addition of CD40 antibody with the adenovirus had the additional benefit of delivering a possible DC maturation signal; both IL-12 production and allo-MLR stimulation were increased in CD40-targeted adenovirus DC preparations (Tillman *et al.*, 1999). Improved gene transfer efficiency was also seen when adenovirus were admixed with liposomes (Dietz and Vuk-Pavlovic, 1998; Tillman *et al.*, 1999). It is not entirely clear why Tillman *et al.* (1999) and Dietz and Vuk-Pavlovic (1998) reported low transduction efficiencies with unmodified adenovirus whereas Zhong *et al.* (1999) and Arthur *et al.* (1997), among others, reported productive efficiencies approaching 95–100% of human DCs. Concerns over initiation of antiadenovirus immunity were generally not supported by *in vivo* murine data. While adenovirus-infected human DCs could prime an antiviral CTL response after several rounds of *in vitro* stimulation (Butterfield *et al.*, 1998; Perez-Diez *et al.*, 1998), mice immunized with adenovirus-infected DCs did not develop significant antiviral CTLs (Brossart *et al.*, 1997; Kaplan *et al.*, 1999). Furthermore, adenoviral immune mice could still be protected from tumor challenge by immunization with DCs infected by adenovirus encoding TAA transgenes (Kaplan *et al.*, 1999). A paucity of antiadenoviral immunity after immunization with adeno-infected DCs has also been reported by Wan *et al.* (1997). While these results raise concerns that mice may not represent a suitable model for studying *in vivo* effects of

adenovirus-infected DCs (possibly because of a reduced ability to mount an antiviral response compared with humans), these disparate results may also represent differences arising from the priming of CTLs *in vitro* versus *in vivo*. Collectively, it appears that adenovirus (and possibly other viral vectors) represents an efficient means for inserting transgenes into DCs without detrimental effects to its phenotype or function.

#### *Tumor antigen gene-modified DCs capable of priming antitumor immunity*

The feasibility of gene modification of DCs to express TAA was first reported by Alijagic *et al.* (1995). Using peripheral blood monocytes cultured in GM-CSF and IL-4, these investigators were able to transfect the resulting DCs with either a chloramphenicol acetyl transferase (CAT) reporter gene or a gene encoding human tyrosinase, a melanoma TAA. Gene transfection (via cationic lipids) resulted in a fivefold increase in CAT activity, while tyrosinase-transfected DCs elicited T cell clustering and TNF- $\alpha$  release in an antigen-specific CTL clone similar to that achieved using peptide-pulsed DCs (Alijagic *et al.*, 1995). However, neither transfection efficiency nor the effect of transfection on DC phenotype and function (other than activation of a long-term T cell clone) was determined.

Another approach to engineering DCs to express TAA was demonstrated by Reeves *et al.* (1996), who utilized retroviral transduction to deliver the MART-1 melanoma TAA. Since retrovirus most efficiently introduces genes into actively cycling cells, CD34<sup>+</sup> hematopoietic stem progenitor cells (HSCs) as cycling DC precursors were chosen as targets. Using murine CD86 as a marker for viral transfection, 20% transfection efficiency, as determined by flow cytometric analysis of murine CD86-expressing cells, resulted from infection of HSCs. MART-1-expressing DCs were able to stimulate MART-1-specific tumor-infiltrating lymphocytes (TILs) *in vitro*. These DCs could generate a MART-1-specific CTL response from autologous peripheral blood mononuclear cells (PBMCs), suggesting that like protein or peptide-pulsed DCs, genetically modified DCs could initiate a specific antitumor T cell response.

In an attempt to improve the efficiency of transgene-expressing DCs, pox and adenoviral vectors capable of directly infecting DCs have been used to express TAA. Infection with either a fowlpox or vaccinia viral vector encoding MART-1 resulted in 50–75% MART-1-expressing DCs (Kim *et al.*, 1997). A single stimulation of peripheral blood lymphocytes (PBLs) from some melanoma patients with MART-1 transgene-expressing DCs but not with MART-1 peptide-pulsed DCs resulted in antigen-specific IFN- $\gamma$  production and CTL generation. Full processing of the whole MART-1 protein and presentation by the DCs was confirmed by generation of CTLs against multiple MART-1 epitopes. Other investigators using an adenoviral vector encoding full-length MART-1 also reported the generation of MART-1-specific CTLs by virally infected DCs (Butterfield *et al.*, 1998; Perez-Diez *et al.*, 1998). MART-1-expressing DCs (but not DCs infected with empty vector) stimulated IFN- $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells from many melanoma patients and healthy donors as measured by bulk enzyme-linked immunosorbent assay (ELISA) and intracellular cytokine staining (Perez-Diez *et al.*, 1998). Interestingly, no IL-4-producing T cells arose from coculture of

MART-1-expressing DCs with bulk PBLs, suggesting a bias towards generation of a helper T cell type 1 (Th1) response by TAA gene-modified DCs (Perez-Diez *et al.*, 1998). TAA-specific CTLs or IFN- $\gamma$  production could not be elicited by virally infected DC-stimulated peripheral blood in all melanoma patients. Also of note, adenovirus-specific CTLs and IFN- $\gamma$  production were present in PBL cultures after repeated stimulation with adeno-infected DCs, raising a concern about the efficacy of virally infected DCs *in vivo* (see above) (Butterfield *et al.*, 1998; Perez-Diez *et al.*, 1998). With those caveats in mind, however, these data suggest that DCs expressing a full-length TAA protein were capable of processing and presenting TAA via both the MHC class I and class II pathways and could elicit superior T cell stimulation compared with peptide-pulsed DCs.

Mouse tumor models have been used to test the *in vivo* therapeutic efficacy of TAA gene-modified DCs. Early efforts centered on tumor lines altered to express a model "tumor" antigen such as  $\beta$ -galactosidase ( $\beta$ -Gal) or ovalbumin (OVA) and DCs expressing  $\beta$ -Gal or OVA transgenes (Brossart *et al.*, 1997; Song *et al.*, 1997; Specht *et al.*, 1997; Wan *et al.*, 1997; McArthur and Mulligan, 1998; De Veerman *et al.*, 1999). Both retroviral (Specht *et al.*, 1997; De Veerman *et al.*, 1999) and adenoviral (Brossart *et al.*, 1997; Song *et al.*, 1997; Wan *et al.*, 1997; McArthur and Mulligan, 1998) transduction systems have been used to insert genes encoding OVA or  $\beta$ -Gal into DCs derived from bone marrow or splenic precursors or immortalized DC lines. The resulting DCs generally showed no alterations in phenotype as measured by flow cytometric analysis of T cell stimulatory molecules such as MHC I/II, CD80/CD86, and ICAM-1 (De Veerman *et al.*, 1999). Genetically modified DCs were able to activate antigen-specific T cell lines *in vitro* (Brossart *et al.*, 1997; De Veerman *et al.*, 1999), generate CTLs *in vivo* (Brossart *et al.*, 1997; Song *et al.*, 1997; Specht *et al.*, 1997; Wan *et al.*, 1997), and promote antitumor immunity in both naive and tumor-bearing mice (Brossart *et al.*, 1997; Song *et al.*, 1997; Specht *et al.*, 1997; Wan *et al.*, 1997; De Veerman *et al.*, 1999). In some studies peptide-pulsed and gene-modified DCs were compared for their ability to initiate T cell activation (both *in vitro* and *in vivo*) and antitumor immunity (Brossart *et al.*, 1997; Specht *et al.*, 1997). DCs infected with either an adenovirus or vaccinia virus encoding a CTL epitope of OVA were equivalent to OVA protein or peptide-pulsed DCs in their ability to activate an OVA-specific CD8<sup>+</sup> T cell line, prime OVA-specific CTLs from naive mice, and generate CTLs on immunization in naive mice (Brossart *et al.*, 1997). More importantly, virally (either adenovirus or vaccinia) transduced DCs generated protective antitumor immunity in both naive mice or mice previously infected with the corresponding virus, suggesting that antiviral immunity does not affect the efficacy of transfected DCs, at least in mice. Using DCs generated from retrovirally transduced bone marrow precursors, Specht *et al.* (1997) showed that genetically engineered DCs could promote therapeutic antitumor immunity in a metastatic model of disease. Treatment of day 3 lung metastases of a  $\beta$ -Gal-expressing CT26 colon carcinoma cell line was equally effective in reducing tumor load using either peptide-pulsed or gene-transduced DCs; however, gene-transduced DCs were more potent primers of  $\beta$ -Gal CTLs *in vivo*. In another interesting mouse model, NFSA murine fibrosarcoma cells engineered to express the MART-1 human melanoma antigen were rejected in mice

immunized with DCs transfected with a MART-1 gene-encoding adenovirus (Ribas *et al.*, 1997). While these results are suggestive, tumor cells expressing novel antigens are rendered more immunogenic compared with the parental cell line, which poses the question of whether these results are due not to potential antigen presentation by DCs but rather arise from the artificial nature of the tumor model per se.

To that end, studies have addressed the question of whether DCs genetically engineered to express naturally occurring TAA are capable of eliciting antitumor immunity *in vivo*. Tuting *et al.* (1997) inserted a plasmid encoding wild-type p53 peptide into bone marrow-derived DCs via a particle bombardment or "gene gun" technique. This biolistic approach yielded a 5–10% transfection efficiency and transgene expression lasting less than 72 hr (Tuting *et al.*, 1997). Nevertheless, immunization of naive mice with p53-transfected DCs resulted in protective immunity from a CMS4 sarcoma, which is known to overexpress p53 (Tuting *et al.*, 1997). However, CMS4 is a chemically induced tumor, which is generally more immunogenic than naturally arising tumors found in humans. In another mouse model, immunization of naive mice with DCs transduced with an adenovirus encoding human p53 resulted in protection of >70% of mice challenged with tumors expressing human p53 or those expressing mutated murine p53 (Ishida *et al.*, 1999). Another report has shown that DCs infected with adenoviral vectors encoding different TAA expressed by the murine melanoma line B16 could elicit antitumor immunity to this naturally occurring and poorly immunogenic tumor model (Kaplan *et al.*, 1999). In this study, DCs infected by adenovirus showed a 95% transfection efficiency and no alterations in phenotype, with the exception of an increase in MHC class I expression. When expressed by DCs, target genes encoding CTL epitopes of tyrosinase-related protein 2 (TRP-2), or human gp100 (hgp100), but not mouse gp100, elicited nearly complete protective immunity in immunized mice subsequently challenged with viable tumor cells. Immunization with TRP-2- and hgp100-expressing DCs could also slow the growth of recently implanted tumors; combination of both TAA-expressing DCs further enhanced the antitumor effect. Although encouraging, established tumors could not be completely eliminated after vaccination with TAA-expressing DCs even though treatment began 4 days after tumor challenge, before a palpable tumor arises (Kaplan *et al.*, 1999). This limitation may be due to the fact that only CTL epitopes were encoded by the transgene and that the DCs could not fully initiate CD4<sup>+</sup> T cell activation in the absence of helper epitopes, a requisite for antitumor immunity (Toes *et al.*, 1999). These translational studies extend the *in vitro* data using genetically modified human DCs to stimulate antitumor immunity to well-characterized animal models. The results to date suggest that TAAs are suitable transgenes for use in immunotherapy protocols (Table 2). However, since most of these models utilize artificially generated tumor antigens or treated mice with small tumor loads (and did not result in curative treatment), further modification of the system will need to be carried out to improve therapeutic results.

#### Cytokine gene-modified DCs

Besides genetic modification to express TAA, DCs have been successfully gene modified to express immunomodulatory



TABLE 2. TUMOR ANTIGEN GENE-MODIFIED DCs

Species	Antigen (transfection vector)	Comments	References
Human	Tyrosinase (liposomes)	Transfected Dc-activated Ag-specific T cell line	Alijagic <i>et al.</i> (1995)
	MART-1 (viral vectors)	Superior to peptide-pulsed DCs in eliciting CTLs Primed CD4 <sup>+</sup> and CD8 <sup>+</sup> T cell response Primed antiadenoviral CD8 <sup>+</sup> T cell response	Kim <i>et al.</i> (1997); Butterfield <i>et al.</i> (1998); Perez-Diez <i>et al.</i> (1998)
Mouse	Multiple melanoma TAA (gene gun)	Improved priming of CD8 <sup>+</sup> T cell response by cotransduction with IL-12 or IFN- $\alpha$	Tuting <i>et al.</i> (1998)
	Ovalbumin, $\beta$ -galactosidase, human MART-1 (viral vectors)	Immunization promoted protection and therapeutic antitumor immunity	Brossart <i>et al.</i> (1997); Ribas <i>et al.</i> (1997); Specht <i>et al.</i> (1997) <sup>a</sup>
	Human p53 (adenovirus)	Highly immunogenic model "tumor" antigen Protected mice from challenge with human p53-expressing tumors and mutant murine p53 tumors	Tuting <i>et al.</i> (1997)
	Murine p53 (gene gun)	Protected mice from challenge with CMS4 sarcoma	Ishida <i>et al.</i> (1999)
	Tyrosinase-related protein 2, human gp100 (adenovirus)	Inhibited growth of established B16 melanoma	Kaplan <i>et al.</i> (1999)

<sup>a</sup>See text for complete references.

proteins such as cytokines and chemokines (Table 3). One disadvantage in selecting TAA as a target gene for modification of DCs is the tissue and MHC haplotype restriction of whole tumor proteins and CTL epitopes, respectively. But DCs modified to express genes encoding T cell stimulatory cytokines, for example, may be potentially used as adjuvants to treat any number of tumors, so long as a source of TAA is available. Cytokine gene-modified DCs represent a potentially more potent vaccine than similarly gene-modified tumor cells since the former are both APC and cytokine factories while the latter require host APC function (Tepper and Mule, 1994).

In murine models of melanoma and sarcoma, IL-12 gene-modified DCs injected directly into tumors induced a profound antitumor response (Nishioka *et al.*, 1999). While the amount of tumor growth inhibition correlated with IL-12 production by the DCs, the effect was dependent on DC stimulation of tumor-specific immunity because IL-12 gene-modified fibroblasts injected intratumorally had little effect on tumor growth (Nishioka *et al.*, 1999). Tuting *et al.* (1998) reported that IL-12 gene-modified DCs could boost priming of TAA-specific CD8<sup>+</sup> CTLs *in vitro*. In this study, DCs were genetically modified to express different melanoma TAAs as well as either IL-12 or IFN- $\alpha$ . IL-12 gene modification has also been shown to boost CD4<sup>+</sup> T cell responses in infectious disease models. CD34<sup>+</sup>

DCs derived from peripheral blood and retrovirally transduced with genes to produce either IL-12 or IFN- $\gamma$  could augment CD4<sup>+</sup> T cell-mediated cytokine production in response to bacterial antigens (Ahuja *et al.*, 1998). Furthermore, immunization of naive mice with IL-12 gene-modified DCs loaded with soluble antigens from *Leishmania donovani* provided better protection from leishmaniasis than antigen-loaded unmodified DCs (Ahuja *et al.*, 1999). In both the mouse and human studies, IL-12 gene transfer led to DC maturation, as measured by increased MHC class II and costimulatory molecule expression (Tuting *et al.*, 1998; Nishioka *et al.*, 1999). Together, IL-12 gene modification of DCs could augment priming to antigens delivered to DCs in a variety of manners and that these DC could represent powerful adjuvants for activation of both CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> CTLs.

Additional cytokine genes have been introduced into DCs with the intent of increasing adjuvanticity. Retroviral infection of PBMCs with an IL-7 gene construct followed by maturation with GM-CSF plus IL-4 resulted in a DC population with increased stimulatory capacity compared with untransfected cells (Westermann *et al.*, 1998). Presumably, the increased proliferation seen in allogeneic and autologous MLR was due to IL-7 enhancement of T cell proliferation. However, MLR assays do not address how cytokine expression of DCs affects antigen-

TABLE 3. CYTOKINE GENE-MODIFIED DENDRITIC CELLS

Cytokine	Use of gene-modified DCs (species)	References
GM-CSF	Immunization for treatment of established tumors (mice)	Cao <i>et al.</i> (1999); Curiel-Lewandrowski <i>et al.</i> (1999)
Lymphotoxin	Immunization for treatment of established tumors (mice)	Cao <i>et al.</i> (1999); Zhang <i>et al.</i> (1999)
IL-12	<i>In vitro</i> priming of human T cells Intratumoral injection (mice) Immunization against leishmaniasis (mice)	Ahuja <i>et al.</i> (1998); Tuting <i>et al.</i> (1998) Nishioka <i>et al.</i> (1999) Ahuja <i>et al.</i> (1999)
IL-7	<i>In vitro</i> stimulation of MLR in human PBLs Intratumoral injection (mice)	Westermann <i>et al.</i> (1998) Miller <i>et al.</i> (2000)

TABLE 4. COMPARISONS OF TAA GENE-MODIFIED DCs WITH TAA PEPTIDE-PULSED DCs

Source of DCs	Tumor antigen	Results	Reference
Adherent human PBMCs cultured in GM-CSF + IL-4	Tyrosinase: full-length gene versus tyrosinase peptide (Ty-2)	Equal aggregation of DCs with Ty-2-specific T cell line	Alijagic <i>et al.</i> (1995)
Adherent human PBMCs cultured in GM-CSF + IL-4	MART-1: full-length cDNA versus MART-1 <sub>27-35</sub> peptide	Increased IFN- $\gamma$ production by CTLs raised by gene-modified DCs	Kim <i>et al.</i> (1997)
Murine bone marrow cells cultured in GM-CSF + IL-4	Ovalbumin: OVA <sub>257-264</sub> cDNA versus OVA <sub>257-264</sub> peptide	Equivalent protective tumor immunity; equivalent CTL generation <i>in vivo</i>	Brossart <i>et al.</i> (1997)
Murine bone marrow cells cultured in GM-CSF + IL-4	$\beta$ -Galactosidase: full-length LacZ cDNA versus $\beta$ -Gal <sub>876-884</sub> peptide	Equivalent protective tumor immunity; superior generation of IFN- $\gamma$ and CTLs <i>in vitro</i> by gene-modified DCs	Specht <i>et al.</i> (1997)

specific or, more specifically, tumor antigen-specific T cell activation. Further experimentation using antigen-specific CTL assays and *in vivo* tests of therapeutic efficacy will be necessary in order to determine if IL-7 gene modification represents a significant improvement on DC-based vaccines. In this regard, one report suggests that IL-7 gene-modified DCs can mediate tumor regression through direct intratumoral injection (Miller *et al.*, 2000). Two reports suggest that modification of DCs with another cytokine cDNA, GM-CSF, can increase therapeutic antitumor immunity *in vivo* as compared with unmodified DCs (Cao *et al.*, 1999; Curiel-Lewandrowski *et al.*, 1999). However, the level of increased antitumor immunity varied between the two studies. In a protective immunity model of a squamous cell carcinoma, 20% of mice immunized with lysate-pulsed, GM-CSF gene-modified DCs (derived from GM-CSF-cultured bone marrow cells) developed a tumor as opposed to 100% of mice treated with unmodified but lysate-pulsed DCs (Curiel-Lewandrowski *et al.*, 1999). It was postulated that increased survival of and migration by GM-CSF gene-modified DCs were responsible for the increased antitumor immunity (Curiel-Lewandrowski *et al.*, 1999). However, in the B16 mouse melanoma model, GM-CSF-expressing splenic DCs fused with tumor cells were only slightly (but not significantly) more effective than unmodified DC/tumor fusions in both protecting mice from tumor challenge and increasing survival of mice with established metastatic disease (Cao *et al.*, 1999). It is unlikely that this discrepancy is due to reported differences in GM-CSF production as Cao *et al.* (1999) reported GM-CSF levels in DC supernatants three to five times the levels reported by Curiel-Lewandrowski *et al.* (1999). More likely, the difference resides in the tissue source of DCs because bone marrow-derived DCs were shown previously to be more potent APCs than splenic DCs and have increased antigen-processing capacity (Fields *et al.*, 1998). However, differences in tumor models and antigen loading may also explain these contradictory results, which may have potential significance for the design of clinical protocols. Finally, DCs genetically engineered to secrete a T cell chemotactic factor, lymphotactin, and subsequently loaded with a peptide of the Mut1 TAA were found to

be potent stimulators of antitumor immunity against the 3LL Lewis lung carcinoma (Cao *et al.*, 1998). In this study, lymphotactin gene-modified DCs were significantly more potent than unmodified DCs. When tumor RNA was used as the source of antigen, lymphotactin-secreting DCs were also superior to untransfected DCs in the induction of antitumor immunity to the B16 melanoma (Zhang *et al.*, 1999), suggesting that this chemokine could be applicable to a range of tumors and antigen sources. Because of the emerging evidence that chemokines play an important role in the priming of naive T cells by DCs (Cyster, 1999), it is likely that future reports will describe the modification of DCs with genes encoding other chemokines.

## CONCLUSION

A number of studies have indicated that genetic modification of DCs can improve their immunostimulatory capacity and provide an efficient means for antigen delivery to T cells. Animal tumor model data suggest that tumor antigen- and cytokine gene-transfected DCs are equally capable and, in many cases, superior APCs relative to unmodified DCs. It is premature at this point to render a decision on whether TAA gene-modified DCs represent a significant improvement in the development of DC-based cancer vaccines since only four studies to date have directly compared the efficacy of TAA gene-transduced DCs with TAA peptide-pulsed counterparts (Table 4). The cumulative results of these studies provide inconclusive evidence to support the hypothesis that TAA gene-expressing DCs are superior APCs relative to TAA peptide-pulsed DCs. Furthermore, tumor lysate- or tumor RNA-pulsed DCs should be compared with TAA gene-modified DCs in order to determine if presentation of a single TAA (via transgene expression) can induce the same level of antitumor immunity as DCs expressing multiple TAAs. However, the results using cytokine gene-modified DCs show, with few exceptions, a significant enhancement of T cell priming and antitumor immunity. An important issue that must still be addressed centers on the multitude of tissue culture protocols employed and different cellular sources from

which DC can be generated. Future work in this field should involve determining optimal conditions and maturation factors for gene-modified DCs. An early indication of this effort comes from a comparison of the timing of antigen pulsing and CD40 maturation in peptide and tumor RNA-loaded DCs (Morse *et al.*, 1998). The effect of CD40-mediated DC maturation on T cell stimulation was found to be most effective when it occurred after peptide pulsing but before loading with tumor RNA. It remains to be seen what temporal restrictions apply to gene modification of DCs with regard to DC maturation protocols. Finally, the question of which target genes are ideally suited for the clinic remains to be fully addressed. Because cytokine production by DCs may enhance antitumor immunity against tumors, it is conceivable that genes encoding cytokines IL-7 and IL-12 or the chemokine lymphotactin may be the first to be used in experimental DC-based vaccines in humans. However, it is also likely that additional cytokines will be shown to augment DC priming of an antitumor response. Another, as yet untested, avenue of research into gene-modified DCs involves expression of T cell adhesion/costimulatory molecules such as B7 or ICAM-1 or DC survival receptors such as CD40 or TRANCE-R. Just as the initial discovery of DC-mediated antitumor immunity prompted excitement for future therapeutics, the early success of gene-modified DCs has engendered another round of optimism.

#### NOTE ADDED IN PROOF

Since the initial submission of this article, several reports have described the use of genetically modified DC. Using retrovirally transduced murine DC encoding full-length OVA protein or a class I MHC restricted epitope of OVA, Schnell *et al.* (2000) provide evidence for a role of CD4<sup>+</sup> T cells in the generation of antitumor immunity against OVA-expressing tumors but not for the initial generation of OVA-specific CTL. Immunization of mice with DC genetically modified to express either full-length human gp100 (Wan *et al.*, 1999) or human MART-1 (Ribas *et al.*, 2000) can protect mice from a lethal challenge of B16 melanoma cells (Wan *et al.*, 1999). MART-1 gene-modified human DC were equivalent to protein loaded counterparts in their ability to stimulate MART-1 specific CTL from melanoma patients (Philip *et al.*, 2000). However, Osterroth *et al.* (2000) report that idiotypic protein-pulsed DCs were far superior to idiotypic gene-modified DCs (via Semlike forest virus) in inducing antigen-specific CTL from PBMC. Finally, Jonuleit *et al.* (2000) report that infection of CD83<sup>+</sup> human DC with adenoviral vectors leads to suppression of the allostimulatory capacity of DC.

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# Enhancement of Tumor Lysate- and Peptide-Pulsed Dendritic Cell-Based Vaccines by the Addition of Foreign Helper Protein

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<sup>1</sup>*Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; B6, C57BL/6J; DC, dendritic cell; KLH, Keyhole limpet haemocyanin; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha; GM-CSF, granulocyte/monocyte colony-stimulating factor; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; TP-DC, tumor lysate-pulsed dendritic cells; TRP, tyrosinase-related protein.

## **Abstract**

We have evaluated whether the addition of a foreign helper protein, keyhole limpet hemocyanin (KLH), can augment the efficacy of tumor lysate-pulsed dendritic cells (TP-DC) and peptide-pulsed DC immunizations in vivo. Besides being used as a "surrogate antigen" in approaches to measure immunologic response in cancer patients, KLH is also an immunogenic carrier protein to elicit T cell help. Using the D5 subline of B16 melanoma, we demonstrate that DC pulsed with both KLH and tumor lysate mediates enhanced immune priming and rejection of established metastases in vivo, which is dependent on host-derived T cells. IL-2 augments the enhancement afforded by KLH, as measured by cure rates and overall survival, in the absence of autoimmune depigmentation. KLH added to DC immunizations markedly enhances tumor-specific T cell production of IFN-gamma. D5 melanoma exposed to similar levels of IFN-gamma results in substantial expression of MHC class I molecules. DC pulsed with KLH and mouse TRP-2 peptide results in enhanced reduction of B16 melanoma metastases; the effect is most pronounced in a setting where TRP-2-pulsed DC alone is completely ineffective. Collectively, these findings demonstrate that KLH addition to tumor antigen-pulsed DC immunizations can augment IFN-gamma production and enhance in vivo antitumor activity.

*Key Words:* Immunotherapy, Tumor Vaccines, Dendritic Cells, Foreign Helper Protein, B16 melanoma; T cell help.

## Introduction

The generation of an optimal cytotoxic T cell (CTL) immune response often requires the presence of CD4<sup>+</sup> helper T cells as well as the expression of both helper- and CTL-defined antigen determinants on the same antigen presenting cell (1). Dendritic cells (DC) are known to represent such specialized antigen presenting cells (2,3). DC can induce both the generation and proliferation of specific CTL and T helper cells via simultaneous antigen presentation by major histocompatibility complex (MHC) class I and class II molecules, respectively. We (4-6) and others (7,8) have described the induction of MHC class I- and class II-specific T cell responses following stimulation with tumor antigen(s)-pulsed DC in vitro and in vivo.

Attention is being focused on identifying agents to further enhance T cell reactivity to tumors elicited by DC-based vaccines, particularly with respect to those that can stimulate a potent T helper cell response. Keyhole limpet hemocyanin (KLH) is one such molecule because it can serve as a helper antigen and can induce a potent specific memory T cell response (9,10). For example, lymph node T cells from mice primed with DC pulsed with KLH can secrete IL-2, IFN-gamma, and IL-4 upon antigen rechallenge in vitro (11). KLH can serve as a strongly immunogenic carrier protein as well. In murine models of idiotypic vaccinations, formulation of syngeneic idiotypic with KLH can induce not only an anti-idiotypic antibody response, but also a T cell proliferative response to idiotypic after priming (12). These findings were originally explained by a "helper effect" whereby a helper determinant introduced onto an antigen presenting cell, ostensibly a DC, can promote an immunologic reaction against that helper determinant. As a consequence, immunologic reactivity to the accompanying tumor-specific antigen(s) increases as well (13). A possible mechanism(s) by which idiotypic-KLH vaccines induces not only humoral responses, but also cell-mediated responses to tumor antigen(s) invokes T cells that recognize idiotypic determinants that have been processed and presented as antigen epitopes by DC, rather than as soluble idiotypic (12).



KLH has an added advantage of being a neo-antigen and can therefore serve as an immunologic tracer molecule in vaccine studies. In this respect, KLH has been shown to serve as a strong "surrogate" antigen and an immunogenic "marker" for immunization studies employing DC-based vaccines (14,15). In addition, KLH when conjugated with idiotype antibody can induce strong humoral responses in vivo, which has resulted in objective outcome in patients with B-cell lymphoma (15).

On the basis of these findings, we have evaluated the capacity of KLH to enhance DC-based tumor vaccines in the setting of the poorly-immunogenic B16 melanoma and its subline, D5. We demonstrate that simultaneous pulsing of DC with KLH and tumor lysates results in pronounced enhancement of vaccine-mediated immune priming and therapeutic efficacy in vivo. The addition of KLH enhances the production of IFN-gamma by tumor-reactive T cells. Vaccine efficacy of tumor lysate/KLH-pulsed DC is augmented further by the systemic administration of IL-2.

## **Materials and Methods**

*Animals.* Six- to 8-week-old female C57BL/6 (denoted B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Animal Maintenance Facility of the University of Michigan Medical Center. The animals were used for experiments between 8 to 14 weeks of age.

*Culture Medium.* Complete medium (CM) consisted of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 µg/mL streptomycin, 100 units/mL penicillin, 50 µg/mL gentamycin, 0.5 µg/mL fungizone (all from GIBCO, Rockville, MD) and  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO.).

*Recombinant Cytokines.* Recombinant murine granulocyte/macrophage-colony factor (GM-CSF) with a specific activity of  $>5 \times 10^6$  units/mg (Immunex, Seattle, WA) and recombinant murine IL-4 with a specific activity  $2.8 \times 10^8$  units/mg (Schering-Plough

Research Institute, Kenilworth, NJ) were used to generate DC (see below). Recombinant human IL-2 (Chiron Corp., Emeryville, CA) with a specific activity of  $18 \times 10^6$  IU/mg protein was administered intraperitoneally to mice. Murine recombinant interferon-gamma (IFN-gamma) was obtained from PharMingen, San Diego, CA (specific activity:  $0.3-1 \times 10^8$  U/mg).

*Tumors.* The B16-BL6 melanoma is of spontaneous origin. A poorly-immunogenic, highly metastatic (accompanied by high CD44<sup>+</sup> cell surface expression) subclone of the B16-BL6 tumor, denoted D5, has been characterized previously (16). The D5 melanoma expresses few to no detectable surface MHC class I molecules and no detectable surface MHC class II molecules. EL-4 is an MHC class II negative, but an MHC class I positive, T cell thymoma syngeneic to C57BL/6 mice.

*Peptide.* Murine TRP-2 (mTRP-2) is a melanosomal membrane glycoprotein expressed on normal melanocytes and B16 melanoma. The H-2K<sup>b</sup>-restricted mTRP-2<sub>181-188</sub> peptide (VYDFFVWL) was synthesized by Research Genetics (Huntsville, AL).

*Generation of Bone Marrow-Derived Dendritic Cells.* Erythrocyte-depleted mouse bone marrow cells were cultured in CM supplemented with 10 ng/mL GM-CSF and 10 ng/mL IL-4 at  $1 \times 10^6$  cells/mL, as described previously (6). On day 6, DC were harvested by gentle pipetting. Harvested cells were then layered onto 14.5% (w/v) metrizamide gradients, centrifuged, and the low-density interface was collected (6). DC were washed twice, enumerated (purity > 80%), and were used for in vitro and in vivo functional studies.

*Antigen Pulsing of Dendritic Cells.* Day 6 DC were incubated with freeze-thawed (3 cycles; centrifuged at 3,500 rpm for 5 min; supernate collected) D5 melanoma lysate at a ratio of 3 tumor cell equivalents to 1 DC (i.e. 3:1) in CM, as described (4,5). In separate experiments, DC were pulsed with mTRP-2 peptide at 10  $\mu$ g/mL for 18 hours. After 18-hr incubation, DC were harvested, washed twice in Hanks' balanced salt solution (HBSS; Gibco) and were resuspended in HBSS for further studies. In some experiments, DC were

co-incubated with tumor lysate or mTRP-2 peptide in the presence of 50  $\mu$ g/mL Keyhole limpet haemocyanin (KLH; subunits 350/400 kDa, endotoxin-free, Calbiochem-Novabiochem Corp., San Diego, CA).

*Interferon-gamma Treatment of D5 Melanoma Cells.* D5 melanoma cells were cultured in CM containing 1,000 pg/mL mouse recombinant IFN-gamma. At 12-hr, the tumor cells were harvested, washed, and analyzed for the expression of cell surface markers by flow cytometry (FACS) after staining with FITC-conjugated anti-I-A<sup>b</sup> and anti-H-2K<sup>b</sup> mAb; comparisons were made with appropriate isotype-matched control mAb (all from PharMingen).

*Primary Immunization.* Normal B6 mice were immunized three times at 7-day intervals with KLH- plus D5 tumor lysate-pulsed DC (KLH/TP-DC). IL-2 was given i.p. twice daily at 60,000 IU in 0.5 mL HBSS for 5 days consecutively after each immunization. Control groups of mice received either no treatment (HBSS), or D5 tumor lysate-pulsed DC (TP-DC), with or without KLH pulsing, and/or with or without IL-2 administration (as detailed in Figures). Mice were rechallenged with  $5 \times 10^4$  viable D5 melanoma cells and were then followed for survival as recorded as the percentage of surviving animals over time (in days) after tumor injection. Data are recorded from five or more mice/group. In separate experiments, B6 mice were immunized with mTRP-2 peptide-pulsed DC (mTRP-2-DC) twice at 7-day intervals and then rechallenged with  $1 \times 10^5$  B16 melanoma cells. Survival was followed as described above.

*Treatment of Established Pulmonary Metastases.* B6 mice received  $1 \times 10^5$  viable D5 melanoma cells intravenously (i.v.) in the lateral tail vein to establish pulmonary metastases, as described (6, 17-19). The mice were then immunized s.c. with  $1 \times 10^6$  KLH/TP-DC three times on days 3, 7, and 11 after tumor injection. IL-2 was given i.p. twice daily at 60,000 IU in 0.5 mL HBSS for 3 days consecutively after each immunization. Control groups of mice received either no treatment (HBSS), D5 tumor lysate, unpulsed DC, TP-DC, with or without KLH pulsing, and/or with or without IL-2 administration (as

detailed in Figures). On day 15 after tumor injection, pulmonary metastases were enumerated in a blinded, coded fashion after insufflation and fixation of the lungs with Fekette's solution, as described previously (6, 17-19). Data are reported as the mean number of metastases  $\pm$  SEM; five or more mice/group. In separate experiments, B6 mice harboring pulmonary micrometastasis were immunized with mTRP-2-DC and pulmonary metastases were enumerated, as described above.

*Antibody Depletion of T Cell Subsets.* In a 3-day pulmonary metastases model, on days -4 and -1 before the first immunization, groups of mice received 200  $\mu$ L of ascites antibody i.v. (GK1.5, 2.43, or rat IgG) to deplete of CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Antibody treatment continued on days 2, 6, and 10 to ensure chronic depletion of the desired cell type, as described (6). The efficacy of depletion was analyzed by FACS and determined to be 99-100% effective (data not shown; (6)). On day 15 after tumor injection, splenocytes were prepared for analysis of IFN-gamma production by standard ELISA (see below).

*IFN-gamma assays.* Erythrocyte-depleted splenocytes ( $2 \times 10^6$  cells/ml) were cultured for 48-hr in vitro with  $2 \times 10^5$  UVB-irradiated D5, or EL-4 tumor cells in 24-well culture plates. After 48-hr, culture supernatants were collected for measurement of murine IFN-gamma release by standard ELISA (PharMingen).

## Results

*Keyhole Limpet Haemocyanin (KLH) Can Enhance DC-based Immunization to Protect Mice from a Lethal Challenge Dose of Melanoma.* We first examined whether or not KLH could enhance immune priming of mice to the poorly-immunogenic D5 subline of B16 melanoma by TP-DC. As shown in Figure 1, mice receiving TP-DC alone showed 20% protection from tumor challenge and these mice survived over 100 days (mean survival time; MST=58 $\pm$ 7,  $p < 0.001$ ; compared to mice receiving HBSS). Immunization with KLH/TP-DC resulted in further protection from tumor challenge; 40% of these mice survived over time (MST=70 $\pm$ 9) and were rendered disease-free. Combination of low-dose

IL-2 and TP-DC also resulted in enhanced protective immunity compared to TP-DC alone, as reported previously in our other tumor models (20). Forty-percent of these mice experienced tumor free survival as well (MST=64±8). In contrast, the combination of KLH/TP-DC and IL-2 resulted in slower tumor growth rate and eighty-percent of these mice survived long-term and were rendered tumor-free (MST=90±6,  $p=0.006$ ). These cured mice were rechallenged 150 days after tumor inoculation with  $3 \times 10^5$  D5 melanoma cells (i.e. a three-fold higher challenge dose); all showed complete protection. An additional cohort of the cured mice were challenged with EL-4 cells and showed no protection to this unrelated tumor (data not shown). These findings indicate that KLH can enhance the efficacy of TP-DC plus IL-2 to protect mice from lethal challenge with the poorly-immunogenic D5 melanoma. In no immunized animal was autoimmune depigmentation observed.

*KLH Can Enhance the Therapeutic Efficacy of DC-based Immunization Against Established Tumor.* To determine the therapeutic potential of immunization with KLH/TP-DC, we attempted to induce tumor rejection in mice with established D5 melanoma. In a 3-day treatment model, B6 mice harboring pulmonary micrometastases were treated with TP-DC with or without KLH. As shown in Table 1, mice receiving TP-DC showed partial reduction (approximately 25%) in the number of pulmonary metastases (mean=166±11,  $p<0.0001$ ; compared to mice receiving HBSS). Treatment with the combination of TP-DC and IL-2 resulted in a further reduction (approximately 55%) in the number of lung nodules (mean=100±11,  $p<0.0001$ ; compared to mice receiving TP-DC). All mice treated with KLH/TP-DC had fewer than 80 nodules (mean=56±5,  $p<0.01$ ; compared to mice treated with TP-DC and IL-2). Furthermore, combined treatment with KLH/TP-DC and IL-2 resulted in a significant reduction (90%) in the number of pulmonary metastases (mean=16±7,  $p<0.01$ ; compared to mice receiving KLH/TP-DC). Of importance, 9 of 15 mice experienced complete tumor regression. In no animal was autoimmune depigmentation elicited during and after treatment. Depletion of either CD4<sup>+</sup> or

CD8<sup>+</sup> T cells by antibody treatment, as described in Materials and Methods, abrogated the efficacy of KLH/TP-DC to regress tumor nodules (Table 2), indicating that the host-derived mechanism of tumor regression mediated by DC immunizations involved both T cell subsets. Collectively, these findings demonstrated that KLH enhanced the therapeutic efficacy of DC immunizations as well as protective immunity against a poorly-immunogenic tumor, which could be enhanced farther by the systemic administration of IL-2.

*KLH Can Augment Tumor-Specific IFN-gamma Production.* We and others have shown previously that tumor-specific IFN-gamma production by host-derived T cells has correlated with antitumor responses in vivo (21,22). On the basis of these findings, we hypothesized that enhancement of the efficacy of DC immunization by KLH may correlate with IFN-gamma production by primed immune cells. To address this possibility, we next evaluated whether or not KLH/TP-DC could elicit tumor-specific IFN-gamma production in vivo. As shown in Figure 2, splenocytes from mice treated with TP-DC and IL-2 produced greater amounts of IFN-gamma ( $13,700 \pm 1,700$  pg/mL;  $p < 0.05$ ) in response to relevant tumor than those receiving TP-DC ( $4,800 \pm 500$  pg/mL). In addition, splenocytes from mice treated with KLH/TP-DC resulted in a further significant IFN-gamma production ( $20,200 \pm 2,800$  pg/mL;  $p < 0.01$ ) compared to mice receiving TP-DC, which was augmented over two-fold by the inclusion of IL-2 ( $51,200 \pm 11,000$  pg/mL;  $p < 0.05$ ). Splenocytes from the control groups of mice produced undetectable levels of IFN-gamma ( $< 97.5$  pg/mL). Thus, IFN-gamma production correlated with in vivo antitumor activity (Table 1). In contrast, splenocytes from treated mice showed little, if any, detectable levels of IL-4 production (data not shown), indicating that DC immunization with or without KLH pulsing induced a Th1 immune response in vivo. As shown in Table 3, in vivo depletion of host-derived CD4<sup>+</sup> T cells completely abrogated IFN-gamma production by the harvested splenocytes ( $1,100 \pm 100$  pg/mL), demonstrating that IFN-gamma production in vivo was indeed CD4-dependent. We also evaluated IFN-gamma production by splenocytes from

mice treated with KLH/TP-DC in response to KLH protein stimulation. Splenocytes from the immunized mice were stimulated with either irradiated D5 melanoma cells or KLH protein in vitro. At 48-hr, supernatants were collected. The cells produced IFN-gamma specifically in response to stimulation by KLH protein ( $9,729 \pm 588$  pg/ml;  $p < 0.001$ ) as well as to the D5 melanoma, indicating that TP-DC with KLH pulsing may "educate" naïve T cells capable of responding to KLH as well as to tumor antigen(s), respectively.

*Surface Phenotype of Parental and IFN-gamma Treated D5 Melanoma Cells.*

Because IFN-gamma production correlated with in vivo antitumor activity, we determined whether or not IFN-gamma produced by effector cells in vivo may influence characteristics of D5 melanoma cells. As shown in Figure 3, D5 melanoma cells expressed only very low levels of surface MHC class I molecules and no detectable surface MHC class II molecules. In contrast, IFN-gamma exposure of D5 melanoma cells resulted in a substantial upregulation of MHC class I (Figure 3). Expression of surface MHC class II and CD95/Fas was low or absent on D5 melanoma cells with or without IFN-gamma (data not shown).

*KLH Can Enhance the Therapeutic Efficacy of DC Pulsed with the H-2K<sup>b</sup>-Restricted, TRP-2 Peptide.*

We next determined the effect of added KLH on the capacity of peptide-pulsed DC to impact on established B16 melanoma. We employed the H-2K<sup>b</sup>-restricted mTRP-2<sub>181-188</sub> peptide expressed by the parental B16 melanoma. As an initial step, B6 mice were first immunized s.c. twice with  $1 \times 10^6$  mTRP-2-DC; control groups of mice received HBSS, unpulsed DC, or irradiated B16 tumor cells to ascertain the capacity of this peptide to prime mice to effectively reject viable tumor cell challenge. All mice were then rechallenged with  $1 \times 10^5$  viable B16 melanoma cells. As shown in Figure 4, mice receiving mTRP-2-DC showed complete protection from tumor challenge ( $p < 0.0001$ ). These tumor-free mice were rechallenged 90 days after tumor inoculation with  $3 \times 10^5$  B16 melanoma cells (3-fold higher number than previous challenge), or with EL-4 tumor cells. All mice showed complete protection from rechallenge with the former but not the latter tumor cells

(data not shown). These initial findings indicated that immunization with mTRP-2-DC elicited strong protective immunity as well as memory response to B16 melanoma.

Given these data, we then evaluated the efficacy of mTRP-2-DC to induce therapeutic rejection of established B16 melanoma. B6 mice received  $2 \times 10^5$  B16 melanoma cells i.v. and then were immunized with  $1 \times 10^6$  mTRP-2-DC with or without KLH pulsing on days 4 and 8 after tumor injection. As shown in Table 4, treatment with mTRP-2-DC alone resulted in no reduction in the number of established pulmonary metastasis (mean=250). Thus, although mTRP-2-DC could effectively prime mice to reject a lethal challenge of viable B16 melanoma cells, the vaccine by itself failed to have any impact on established tumor. Combination of mTRP-2-DC and IL-2 demonstrated modest, but significant reduction in the number of pulmonary metastases (mean=220 $\pm$ 3,  $p < 0.001$ ; compared to mice receiving mTRP-2-DC alone). In contrast, mice treated with KLH/mTRP-2-DC experienced partial reduction (25%) in the number of pulmonary nodules (mean=188 $\pm$ 7,  $p < 0.01$ ). Of importance, IL-2 administration further enhanced the efficacy of KLH/mTRP-2-DC to regress pulmonary nodules by 60% (mean=102 $\pm$ 10,  $p < 0.01$ ).

## Discussion

We reported previously that the systemic administration of IL-2 could potentiate the antitumor effects of TP-DC in vivo during both primary immunization and treatment of established tumors in murine models of a weakly-immunogenic fibrosarcoma and breast cancer (20). To further determine the therapeutic efficacy of the combination of TP-DC and IL-2 on other histologically distinct murine tumors that differ in levels of inherent immunogenicity, we employed the poorly-immunogenic B16 melanoma and its subline, D5, which express few to no detectable surface MHC class I molecules and no detectable surface MHC class II molecules.

In the current study, the systemic administration of IL-2 at relatively high doses (60,000 IU/dose) enhanced TP-DC-based immunizations to promote protective immunity



toward, and therapeutic rejection of the B16 melanoma and its subline, D5. Thus, unlike in our previous work with sarcoma and mammary tumor, treatment of these melanomas by TP-DC plus IL-2 required higher doses of IL-2 to promote antitumor activity. Attempts to escalate the IL-2 dose beyond 60,000 IU to achieve greater antitumor effects when combined with TP-DC immunization were not possible because of dose-limiting toxicity. Toxicity from high-dose IL-2 administration has been reported previously in both animals and humans, which has hampered its wider use as an agent in cancer therapy (23-25). Given this limitation, we attempted to combine IL-2 with a foreign helper protein.

We demonstrated that KLH, a strongly immunogenic carrier protein, could augment the efficacy of tumor lysate- or TRP-2 peptide-pulsed DC immunization in mediating both successful immune priming toward and therapeutic rejection of the B16 melanoma and its subline, D5. These effects could be further enhanced by the systemic administration of IL-2 and were dependent on host-derived CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells from mice treated with KLH/TP-DC could secrete elevated amounts of the Th1-type cytokine, IFN-gamma, in a specific fashion, after exposure to melanoma cells. Further, IFN-gamma exposure of the melanoma cells resulted in a marked upregulation of MHC class I molecules.

The level of expression of MHC class I molecules on tumor cells is an important determinant of their interaction with CTL (26,27). A variety of cytokines have been shown to augment MHC expression on tumor cells in vitro (28,29). Moreover, Weber et al. (27,30) demonstrated that in vivo treatment of mice bearing s.c. B16 melanoma with the systemic administration of recombinant IFN-gamma could up-regulate tumor expression of MHC class I but not class II molecules. This process resulted in both an enhanced sensitivity of the B16 melanoma to treatment with recombinant IL-2 and the generation of specific, therapeutic tumor-infiltrating lymphocytes. In our previous study (20), we demonstrated that splenocytes from mice immunized with TP-DC could produce IFN-gamma (>2,000 pg/mL) when stimulated in vitro with relevant TP-DC. Based on these findings, we sought to determine whether IFN-gamma could up-regulate the expression of MHC molecules on

melanoma cells. Indeed, surface expression of MHC class I molecules by D5 melanoma cells was strikingly elevated by relatively low-dose (1,000 pg/mL) IFN-gamma treatment in vitro.

With respect to the use of B16 and D5 melanoma lysates as the source of antigen(s) for pulsing of DC, in no case of primary immunization nor treatment of established tumors did we observe any animal develop autoimmunity as manifested by depigmentation following the elicitation of potent antitumor immunity in vivo. This finding is seemingly at odds with those reported by others in which mice immunized with either TRP-1 (31,32) or intact B16 melanoma cells (33) developed marked depigmentation with successful tumor immunity. The complete lack of induction of depigmentation by TP-DC immunization in our experience might be explained by differences in the strategies employed for immunization (e.g., DC vs. recombinant vaccinia virus, DNA, or GM-CSF and CTLA-4 blockade) or by the lack of processing and presentation by TP-DC of the antigen(s) responsible for autoimmune reactivity and the induction of autoantibodies or autoreactive CD8<sup>+</sup> T cells. Unlike B lymphocytes, DC have been reported to focus the immune response against select antigenic determinants (34). Alternatively, the density of the particular antigen(s) presented by TP-DC could have affected the outcome of the induced immune response in vivo (35).

Idiotypic-KLH vaccines have been shown to induce not only humoral responses but also cell-mediated responses to tumor antigen(s). These responses were presumably dependent on T cells that recognized idiotype determinants processed and presented as antigen epitopes by DC, rather than as soluble idiotype (10). Based on these studies, we hypothesized that the mechanism(s) by which KLH could augment the therapeutic efficacy of TP-DC-based immunization was dependent on CD4<sup>+</sup> T cells. Indeed, depletion of CD4<sup>+</sup> T cells in mice harboring 3-day pulmonary metastases from the D5 melanoma completely abrogated the efficacy of KLH to augment the effect of DC-based immunization on tumor growth (Table 2). Moreover, production of IFN-gamma was eliminated by the depletion of

CD4<sup>+</sup> T cells (Table 3). These findings corroborate those of Schnell et al. (36), which demonstrated the necessity of CD4<sup>+</sup> T cell help to elicit antitumor in the setting of DC immunization.

Lymph nodes from mice immunized with either KLH protein or KLH-pulsed DC produced IL-2, IFN-gamma, and IL-4 upon antigen rechallenge in vitro (9). Furthermore, almost all IFN-gamma-producing cells co-produced IL-4 (37), indicating that T cells from mice primed in vivo with KLH-pulsed DC were more likely to be skewed towards expression of a Th2 cytokine profile. In contrast, in our current study, CD4<sup>+</sup> T cells from mice immunized with KLH/TP-DC produced greater amounts of IFN-gamma, whereas production of IL-4 was not affected. Coadministration of IL-2 resulted in enhanced production of IFN-gamma but not of IL-4.

Down-regulation of L-selectin (CD62L) on T cells has been shown to occur in vivo during a normal immune response to soluble antigen(s) and to allogeneic cells (38,39). Although the expression of CD62L on lymphocytes is believed to serve a primary function of regulating cell circulation to lymph nodes through the binding to specialized high endothelial venules, the down-regulation of CD62L on T cells also appears to reflect an early physiologic event of T cells responding to antigenic stimulation (40). Within lymph nodes draining a progressively growing tumor, a population of CD4<sup>+</sup> T cells has been shown to differentiate into antigenically-committed immune cells with a concomitant loss of surface CD62L expression (41). Our most recent preliminary data demonstrate that successful immunization of mice with TP-DC increased the population of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells down-regulating CD62L expression (data not shown). Of particular interest, immunization with KLH plus TP-DC resulted in a significant increase in both CD62L<sup>low</sup> CD4<sup>+</sup> and CD62L<sup>low</sup> CD8<sup>+</sup> T cells. In a previous study, anti-CD3/IL-2 activated CD62L<sup>low</sup> cells secreted a number of cytokines, including IFN-gamma, IL-2, IL-4, and IL-10, specifically when stimulated with cognate tumor cells (42). Our findings support the hypothesis that a significant increase in CD62L<sup>low</sup> population of T cells (especially CD4<sup>+</sup> T

cells) by successful immunization of mice with KLH/TP-DC results in the production of greater amounts of the Th1 type cytokine, IFN-gamma, which correlates with their in vivo antitumor reactivity. Moreover, CD62L<sup>low</sup> T cells have shown a high proliferative rate in response to anti-CD3 and IL-2 stimulation (41). Similarly, the combination of KLH/TP-DC and IL-2 demonstrated a further significant increase in CD62L<sup>low</sup> population compared to KLH/TP-DC alone (data not shown).

In our previous studies, we showed that the immune priming and therapeutic activity mediated by TP-DC were dependent predominantly on host-derived CD8<sup>+</sup> and, to a lesser extent, on CD4<sup>+</sup> T cells in both a murine fibrosarcoma and a breast tumor model (6). In contrast, in vivo growth of B16 melanomas could be eradicated by various effector cells such as natural cell-mediated cytotoxicity (42), activated macrophages (43,44), CD4<sup>+</sup> T cells (45), or CD8<sup>+</sup> CTL (44,46-48). In our current study, depletion studies demonstrated that the effect of TP-pulsed DC on pulmonary metastases was critically dependent on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These findings showed that a mixture of tumor lysates and strongly immunogenic carrier protein, KLH, could augment the function of CD4<sup>+</sup> T cells to produce a Th1 cytokine, namely IFN-gamma, and generate CD8<sup>+</sup> T cell activity. These results differ from those recently reported by Timmerman and Levy (49). In the latter study, Id-KLH-pulsed DC immunization resulted in a potent elicitation of anti-Id antibodies and tumor regression but surprisingly was not dependent on effector T cells.

Some recent human clinical trials by us (50) and by others (14,15) to evaluate DC-based cancer vaccines have employed the use of KLH in addition to a source of tumor antigen(s) for immunization. In the current study, DC pulsed simultaneously with both KLH and mouse TRP-2 peptide resulted in enhanced reduction of established B16 melanoma metastases. This effect was most pronounced in a setting where TRP-2-pulsed DC immunizations alone were completely ineffective in impacting on established B16 melanoma metastases (Table 4). These results further suggest that DC-based tumor vaccine

strategies may benefit by the addition of KLH, particularly in the setting of CTL-defined tumor peptides.

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## Figure Legends

Figure 1. KLH can enhance the efficacy of DC immunizations to induce protective immunity toward D5 melanoma. B6 mice were immunized s.c. three times with either HBSS, unpulsed DC, or tumor lysate-pulsed DC with or without KLH pulsing, and/or with or without IL-2 administration as described in Materials and Methods. IL-2 was given i.p. twice daily at 60,000 IU for 5 consecutive days after each immunization. The mice were rechallenged 12 days after last immunization with D5 melanoma cells. Survival was monitored over time after tumor inoculation, and the MST (in days) was determined.

Figure 2. KLH can enhance the capacity of tumor lysate-pulsed DC to induce IFN-gamma production. Mice harboring pulmonary micrometastases were treated as described in Materials and Methods. Spleens were harvested 15 days after tumor injection. For measurement of IFN-gamma production, splenocytes from treated mice were stimulated in vitro as described. Culture supernatants were collected 48-hr later and evaluated for IFN-gamma levels by standard ELISA (in pg/mL/2 x 10<sup>6</sup>; mean + SEM of triplicate samples).

Figure 3. Exposure of D5 melanoma cells to IFN-gamma results in upregulation of surface MHC class I molecule expression. D5 melanoma cells were cultured in the presence of 1,000 pg/mL of IFN-gamma for 12 hours. The tumor cells were collected and stained with specific monoclonal antibody (mAb) to MHC class I molecules. In the histograms, the darker line represents staining with the appropriate mAb, and the lighter dotted line represents the isotype control-matched mAb (i.e., background staining).

Figure 4. DC pulsed with mTRP-2<sub>181-188</sub> peptide can elicit protective immunity toward B16 melanoma. B6 mice were immunized s.c. twice with 1 x 10<sup>6</sup> mTRP-2-DC as described in Materials and Methods. Control groups of mice were immunized with either HBSS,

unpulsed DC, or irradiated B16. Seven days after the second immunization, mice were rechallenged with  $1 \times 10^5$  B16 melanoma cells. Tumor growth was measured over time.

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(in press).

Table 1. KLH can enhance the therapeutic efficacy of DC immunizations against established pulmonary metastases

Treatment*					Mean $\pm$ SEM	p $\eta\eta$
HBSS	IL-2	KLH-pulsed DC	TP-DC	Tumor lysate+ KLH-pulsed DC		
+	-	-	-	-	225 $\pm$ 8	-
-	+	-	-	-	228 $\pm$ 7	NS
-	+	+	-	-	235 $\pm$ 10	NS
-	-	-	+	-	166 $\pm$ 11	<0.0001a
-	+	-	+	-	100 $\pm$ 11	<0.0001b
-	-	-	-	+	56 $\pm$ 5	<0.01c
-	+	-	-	+	16 $\pm$ 7	<0.01d

\* Number of D5 melanoma cells injected i.v. was  $1 \times 10^5$ . A total of  $1 \times 10^6$  D5 tumor lysate-pulsed DC with or without KLH pulsing were injected three times on days 3, 7, and 11 after tumor injection. IL-2 was given i.p. twice daily at 60,000 IU/dose for 3 consecutive days after each immunization.

$\eta$  Mice were euthanized at day 15.

$\eta\eta$  One-way factorial ANOVA and Fisher's PLSD were used for statistical analysis.

a, b, c, d; compared to mice receiving HBSS, TP-DC, TP-DC and IL-2, or KLH/TP-DC, respectively.

Table 2. Therapeutic rejection of D5 melanoma by DC-immunization is dependent on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Treatment*		# of Metastases <sup>¶</sup>	Mean $\pm$ SEM	p <sup>¶¶</sup>
Ab Depletion**	Tumor lysate+ KLH-pulsed DC			
—	HBSS	153, 223, 237, 250, 250	223 $\pm$ 18	—
Rat IgG	+	40, 40, 42, 44, 50, 70, 74	51 $\pm$ 5	<0.0001a
anti-CD4	+	109, 153, 194, 241, 250	189 $\pm$ 27	NS a
anti-CD8	+	187, 198, 231, 247, 250	223 $\pm$ 13	NS a

\* Number of D5 melanoma cells injected i.v. was  $1 \times 10^5$ . A total of  $1 \times 10^6$  D5 tumor lysate-pulsed DC with KLH pulsing were injected three times on days 3, 7, and 11 after tumor injection.

\*\* On days -4 and -1 before the first immunization, groups of mice received 200  $\mu$ L of ascites antibody i.v. (GK1.5, 2.43, or rat IgG) to deplete of CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Antibody treatment continued on days 2, 6, and 10, as described in Materials and Methods.

¶ Mice were euthanized at day 15.

¶¶ One-way factorial ANOVA and Fisher's PLSD were used for statistical analysis.

a; compared to mice receiving HBSS



Table 3. Production of IFN-gamma by splenocytes from mice treated with KLH/TP-DC is CD4-dependent.

CD4-depletion in vivo*	Stimulation (IFN-gamma, pg/mL)**		
	D5	EL-4	None
-	20,200±2800	1,200±100	300±60
+	1,100±100	<97.5	<97.5

\* Groups of mice received 200 uL of ascites antibody i.v. to deplete of CD4<sup>+</sup> T cells as described in Legend to Table 2.

\*\* Splenocytes (4 x 10<sup>6</sup>) from treated mice as described in Table 2 were cultured for 48 hr in vitro with 2 x 10<sup>5</sup> UVB-irradiated either D5, or EL-4 cells in 24-well culture plates. After 48-hr, culture supernatants were collected for measurement of murine IFN-gamma release by standard ELISA.

Table 4. KLH can enhance the efficacy of DC pulsed with H-2K<sup>b</sup>-restricted mTRP-2 peptide to induce therapeutic rejection of B16 melanoma

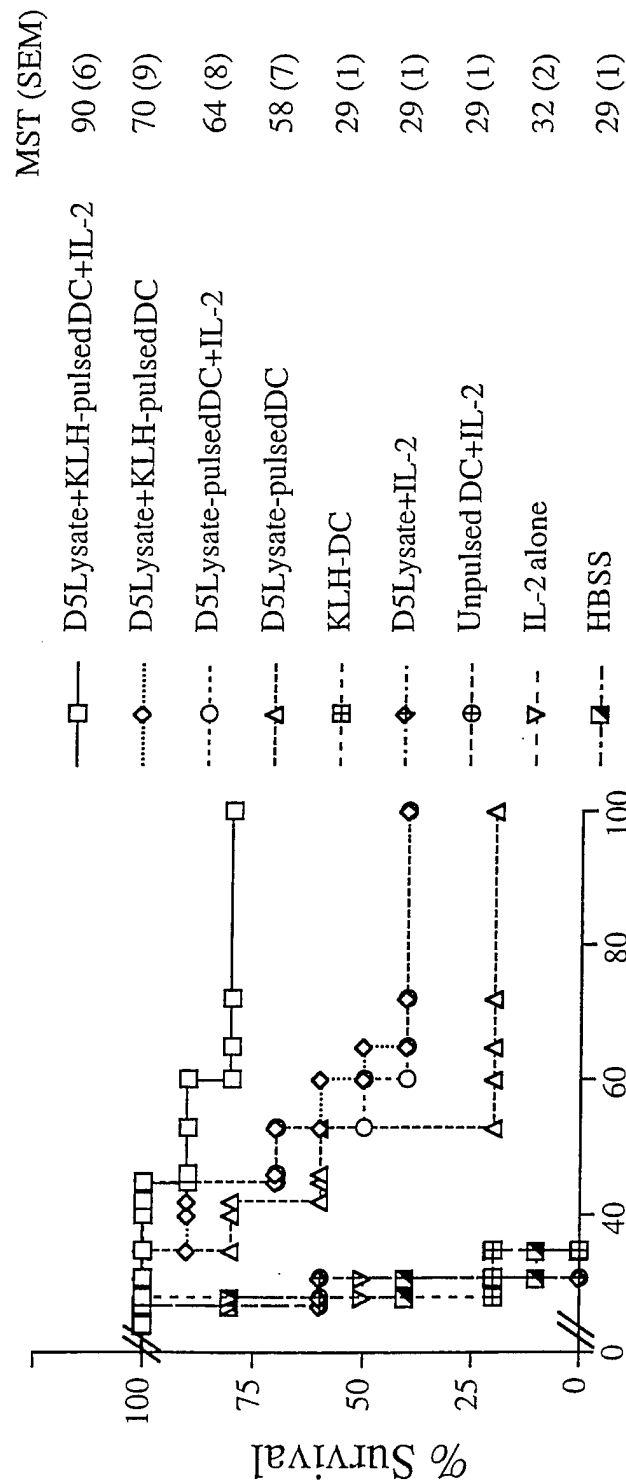
Treatment*					Mean $\pm$ SEM	p <sup>¶¶</sup>
HBSS	IL-2	KLH-pulsed DC	mTRP-2-DC	mTRP-2-plus KLH-DC		
+	-	-	-	-	250	-
					250, 250, 250, 250, 250, 250, 250, 250	
-	+	-	-	-	247 $\pm$ 3	NS
					224, 250, 250, 250, 250, 250, 250, 250, 250	
-	-	+	-	-	243 $\pm$ 5	NS
					212, 216, 250, 250, 250, 250, 250, 250, 250	
-	-	-	+	-	250	NS
					250, 250, 250, 250, 250, 250, 250, 250, 250	
-	+	-	+	-	220 $\pm$ 3	<0.001 <sup>a</sup>
					204, 212, 216, 217, 218, 221, 223, 224, 224, 236	
-	-	-	-	+	188 $\pm$ 7	<0.01 <sup>b</sup>
					127, 186, 186, 191, 196, 196, 196, 199, 200, 202	
-	+	-	-	+	102 $\pm$ 10	<0.01 <sup>c</sup>
					18, 83, 104, 104, 106, 108, 118, 123, 126, 127	

\* Number of B16 melanoma cells injected i.v. was  $2 \times 10^5$ . A total of  $1 \times 10^6$  mTRP-2 peptide-pulsed DC with or without KLH pulsing were injected twice on days 4 and 8 after tumor injection. IL-2 was given i.p. twice daily at 60,000 IU/dose for 3 consecutive days after each immunization.

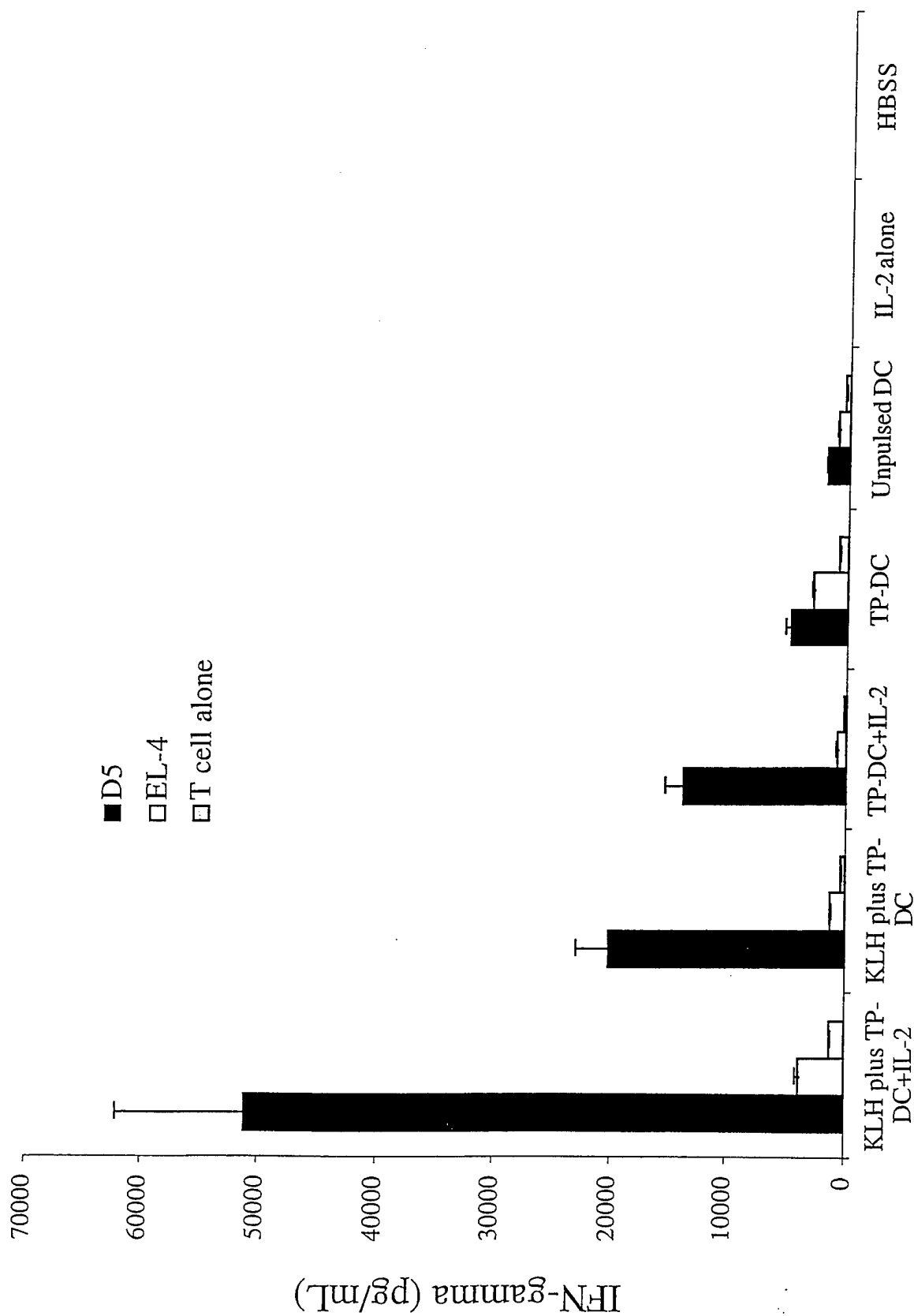
¶ Mice were euthanized at day 14.

¶¶ One-way factorial ANOVA and Fisher's PLSD were used for statistical analysis.

a, b, c; compared to mice receiving mTRP-2-DC, mTRP-2-DC and IL-2, or KLH/mTRP-2-DC, respectively.



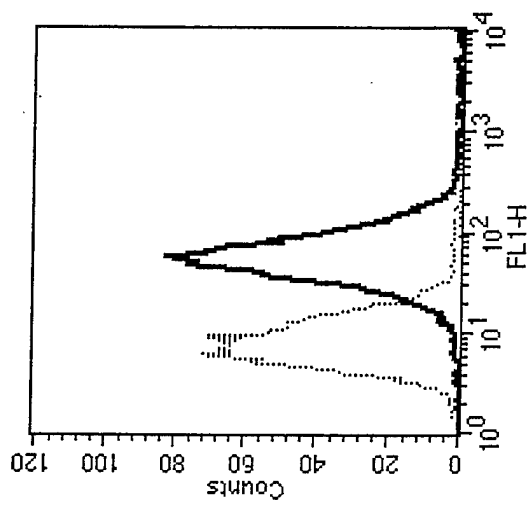
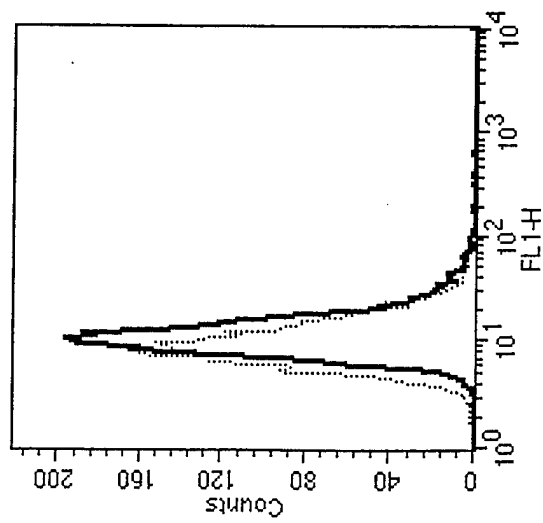
Days after Tumor Injection

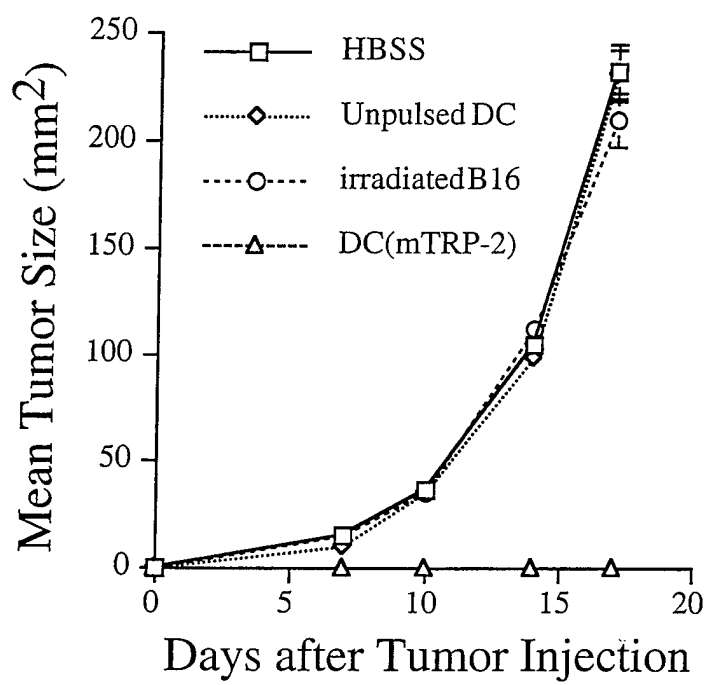


-IFN- $\gamma$

+IFN- $\gamma$

MHC-Class I Expression





**Local Administration of Dendritic Cells Inhibits Established Breast Tumor  
Growth: Implications for Apoptosis-Inducing Agents\***

Running Title: Dendritic Cells Inhibit Breast Tumor Growth In Situ

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<sup>1</sup>Shimizu K., Giedlin M, and Mulé JJ. Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein, Submitted for publication.

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Dendritic cells (DC) can efficiently acquire foreign antigen(s) from apoptotic cells and induce MHC class I-restricted, antigen-specific cytotoxic T lymphocytes (CTL). Accumulation of DC within solid tumor masses in situ has been associated indirectly with a more favorable prognosis. Therefore, DC may offer an efficient means for triggering immune responses within tumors, particularly in those masses containing significant apoptosis. We examined whether delivery of DC could, alone, impact on the progressive growth of a tumor with a relatively high apoptotic index. We detected significant early apoptosis within the mass of a subcutaneously growing murine MT-901 breast carcinoma. DC could efficiently engulf MT-901 tumor apoptotic cells in vitro. Intratumoral injections of syngeneic but not allogeneic DC resulted in significant inhibition of MT-901 tumor growth. Histologic examination of the tumor revealed intense mononuclear cell infiltration during and following DC injections. Tumor growth inhibition was relatively radiosensitive and dependent on host-derived CD8<sup>+</sup> T cells. The baseline level of tumor apoptosis could be increased substantially by TNF- $\alpha$  administration, leading to a greater DC-mediated antitumor effect. The antitumor effect could also be enhanced by first pulsing DC with the foreign helper protein, Keyhole limpet hemocyanin, prior to intratumoral delivery and combining it with the systemic administration of IL-2. Splenocytes from treated animals showed heightened levels of specific CTL activity and production of cytokines. The level of in situ tumor apoptosis appears to play a critical role in DC-mediated antitumor effects. The potential implication of these findings in DC-based tumor therapy strategies is discussed.

Key words: dendritic cells • immunotherapy • T cells • local delivery • apoptosis



## Introduction

Dendritic cells (DC) are potent antigen presenting cells that can both elicit primary and boost secondary immune responses (1-3). Since their original identification by Steinman and colleagues (1,2), much attention is now being focused on the role of DC in eliciting antitumor immunity and in potential therapeutic applications. In this regard, DC pulsed with defined tumor-associated peptides or proteins have been shown to elicit potent antitumor T cell responses both in vitro and in vivo (3-5). We have reported that murine DC can efficiently present antigens associated with whole tumor cell lysates to naive and primed T cells in vitro and can elicit antitumor immunity resulting in tumor regression in vivo (6,7). Moreover, initial clinical trials involving DC-based immunization of patients with tumors of hematologic (8) or solid tumor (9,10) origin have shown promise by generating antitumor T cell reactivity as well as, in some cases, by resulting in partial and complete tumor responses. There is also indirect evidence that suggests the infiltration of solid tumor masses with greater numbers of DC in situ is associated with better prognosis (reviewed in ref. 11). Whether or not this observation directly reflects the induction of an immune response of beneficial consequence in these patients remains to be determined.

It has been shown recently that immature DC can efficiently acquire antigen from apoptotic cells and induce MHC class I-restricted, antigen-specific CD8<sup>+</sup> CTL (12). This finding adds additional support to the concept that DC may play the predominant role in "cross-priming" events for the elicitation of an immune response in vivo (12,13). Bhardwaj and colleagues (14) have shown further that the process of phagocytosis of apoptotic cells requires cell surface expression of  $\alpha_v\beta_5$  and CD36 molecules by the immature DC. Our recent studies have demonstrated that bone marrow-derived DC in early culture are highly active at engulfing high molecular weight dextran particles in vitro (15). Because of these findings, it is conceivable that DC may offer an efficient means for triggering immune responses in situ within tumors, particularly in those masses containing a significant baseline level of apoptotic cell cells.

Our previous therapeutic studies have involved the administration of DC primed with whole tumor lysates (6,7,16). In breast cancer, as example, this approach is difficult, because only rarely has it been possible to isolate enough viable tumor cells from an individual to produce the vaccine. Thus, we have focused our efforts on designing alternative strategies to overcome this potential limitation in DC-based tumor vaccine development. In the current study, we evaluated the effect of intratumoral injections of bone marrow-derived DC on the subcutaneous growth of the murine MT-901 breast tumor, which we show has a prominent baseline level of early apoptosis. We demonstrate that DC can efficiently uptake apoptotic MT-901 tumor cells and that intratumoral injections of DC, alone, can result in regression of this breast tumor in vivo, which is dependent on host CD8<sup>+</sup> T cell immunity. The antitumor and T cell immune effects of locally-delivered DC can be enhanced by either the in vivo administration of a tumor apoptosis-inducing agent, TNF- $\alpha$ , or by pulsing the DC with a foreign helper protein, KLH, in combination with the systemic administration of IL-2.

## **Materials and methods**

**Animals.** Six- to 8-fgfdweek-old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at the Animal Maintenance Facility of the University of Michigan Medical Center. The animals were used for experiments between eight and ten weeks of age.

**Medium and cytokines.** Complete medium (CM) consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 1  $\mu$ M sodium pyruvate, 100  $\mu$ g/mL streptomycin, 100 U/mL penicillin, 50  $\mu$ g/mL gentamicin, and 0.5  $\mu$ g/mL fungizone, all from GIBCO (Grand Island, NY) and 5 x 10<sup>-5</sup> M 2-ME from Sigma Chemical Co. (St. Louis, MO). Recombinant cytokines were used at the following concentrations, diluted in CM: rmGM-CSF, 10 ng/mL

(specific activity:  $\leq 5 \times 10^6$  U/mg) from Immunex Corp. (Seattle, WA); rmIL-4, 10 ng/mL (specific activity:  $2.8 \times 10^8$  U/mg) from Schering-Plough Research Institute (Kenilworth, NJ); rhIL-2, (specific activity:  $18 \times 10^6$  IU/mg) from Chiron Corp. (Emeryville, CA). Recombinant human TNF- $\alpha$  (specific activity:  $8.2 \times 10^6$  U/mg) from Knoll AG, Ludwigshafen, Germany was administered to tumor bearing mice at a single dose of 6  $\mu$ g intravenously.

**Tumors.** MT-7 is a cultured murine tumor cell line derived from a dimethylbenzanthracene (DMB)-induced mammary carcinoma in the BALB/c strain (17). A subline, denoted MT-901, was derived from an early in vivo passage of cultured MT-7 tumor injected subcutaneously (s.c.). This tumor is weakly-immunogenic and expresses MHC class I (but not MHC class II) molecules. Tumors were maintained in vitro followed by one in vivo passage by s.c. injection in syngeneic mice prior to use. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 mL RPMI 1640 containing 8,000 U collagenase (type III; Sigma) for 18-24 hours at 37°C, 65 rpm. The digest was then filtered over sterile 100 nylon mesh (Nytex; TETKO Inc., Briarcliff Manor, NY) and washed three times with brief incubations in Hank's Balanced Salt Solution (HBSS; GIBCO). Renca is an immunogenic murine renal cell carcinoma of spontaneous origin in the BALB/c strain (18,19).

**Detection of apoptotic cells.** Suspensions of MT-901 tumor were prepared at day 8 following the subcutaneous injection of  $5 \times 10^6$  viable cells in BALB/c mice and were analyzed for cells undergoing apoptosis using a standard FACS assay (R & D Systems, Inc., Minneapolis, MN), which detects binding of annexin V-fluorescein and exclusion of propidium iodide (12,20,21). Tumors of mice receiving systemic TNF- $\alpha$  were examined at 24 hr after treatment.

In additional studies, DC that had engulfed apoptotic tumor cells were examined by transmission electron microscopy. DC were fixed in 4% glutaraldehyde in 0.1M cacodylate buffer, pH7.3 for 3 hr. at 4°C. After being washed twice in buffer, the samples were

postfixed in 2% OsO<sub>4</sub> in buffer for 1 hr. at room temperature. The cell pellets were washed 2x in buffer and dehydrated in increasing concentrations of alcohol for 10 min. each to final dehydration in two washes of propylene oxide. The samples were infiltrated with increasing concentrations of epon resin:propylene oxide and finally embedded in pure epon. Thin sections were obtained on an AO Ultracut ultramicrotome, stained with uranyl acetate and lead citrate and viewed on a Philips 400T electron microscope.

**Splenocytes.** Spleen cells obtained from naive BALB/c mice were treated with ammonium chloride-potassium lysis buffer (0.83% ammonium chloride, 0.1% KHCO<sub>3</sub>, 0.004% EDTA) for one minute to deplete erythrocytes and washed twice with HBSS. They were then enumerated and resuspended in HBSS for injection.

**Generation of bone marrow-derived DC.** Erythrocyte-depleted mouse bone marrow cells from flushed marrow cavities of femurs and tibias were cultured in CM supplemented with 10 ng/mL GM-CSF and 10 ng/mL IL-4 at  $1 \times 10^6$  cells/mL, as described previously (15,22). On day 3, DC were harvested by gentle pipetting and were resuspended at  $5 \times 10^6$  cells/mL in CM. Three mL of the DC suspension were overlaid onto three mL of a 14.5% (by weight) metrizamide (Sigma) - CM solution in a 15 mL centrifuge tube. The resulting gradient was centrifuged at 2,000 rpm, brake off, 4°C, for 15 min. The low-density interface containing the DC was collected by gentle pipette aspiration. The DC were washed twice with HBSS, enumerated, and resuspended in HBSS for injection.

**Antigen pulsing of DC.** In some experiments, DC were pulsed with Keyhole limpet hemocyanin (KLH; subunits 350/400 kDa, endotoxin-free, Calbiochem-Navabiochem Corp., San Diego, CA) at 50 µg/mL for 18 hours.

**In vivo treatment of subcutaneous tumor.** BALB/c mice received  $3 \times 10^6$  viable MT-901 tumor cells s.c. on day 0. In some experiments, groups of mice also first received total body irradiation with 500 rad before tumor injection. Except where specifically indicated, all mice were then injected on days 3, 10, 17, and 21 with  $1 \times 10^6$  DC (or normal splenocytes) in two 25 µL intratumoral (i.t.) injections. A control group received HBSS

alone. In other experiments, mice with more established MT-901 tumor received DC i.t. on days 6, 14, and 20 in combination with intravenous (i.v.) TNF- $\alpha$  on days 5 and 13. Control mice received DC, TNF- $\alpha$ , or HBSS alone. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters with calipers, as described previously (23). Data are reported as the average tumor area  $\pm$  SEM.

**Allogeneic DC injection.** Normal BALB/c mice were injected s.c. in the right flank with  $1 \times 10^6$  MT-901 tumor cells. At day 14 after injection, the mice received i.t. with either  $2 \times 10^6$  unpulsed allogeneic DC in 50  $\mu$ L (derived from C57BL/6),  $2 \times 10^6$  unpulsed syngeneic DC (derived from BALB/c), or HBSS. The tumor size was measured as described above.

**Depletion of CD8<sup>+</sup> T cells and treatment of subcutaneous tumor.** BALB/c mice were depleted of CD8<sup>+</sup> T cells by 200  $\mu$ L intravenous (i.v.) injection of anti-CD8 (2.43, rat IgG2b) monoclonal ascites antibody (American Type Culture Collection, Rockville, MD) on days 0, 7, 14, and 21, as described previously (24). Control mice received rat IgG (Sigma) for isotype control of antibody function. The efficacy of depletion was analyzed by FACS and determined to be 99-100% effective (24; data not shown). On day 0, all mice received  $3 \times 10^6$  viable MT-901 tumor cells s.c. Mice receiving either anti-CD8 or rat IgG antibody were then also injected on days 3, 10, 17, and 21 with  $1 \times 10^6$  DC in two 25  $\mu$ L i.t. injections. Control groups received HBSS injections. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters with calipers, as described previously (23). Data are reported as the average tumor area  $\pm$  SEM.

**Treatment of subcutaneous tumor with KLH-pulsed DC.** Normal BALB/c mice received  $5 \times 10^5$  viable MT-901 tumor cells s.c. on day 0. The mice were then injected on days 7, 10, 13, 17 and 20 with  $2 \times 10^6$  DC in a 50  $\mu$ L i.t. injection. Control groups of mice received either unpulsed DC ( $2 \times 10^6/50 \mu$ L), normal splenocytes ( $2 \times 10^6/50 \mu$ L), or

HBSS alone. IL-2 was given intraperitoneally (i.p.) twice daily at 60,000 IU in 0.5 mL HBSS for 2 days after each treatment. The size of the tumor was assessed and survival was followed as recorded as the percentage of surviving animals over time (in days).

At day 120 after tumor injection, the mice that had experienced complete tumor elimination were rechallenged with  $1 \times 10^6$  viable MT-901 tumor cells in the left flank and  $5 \times 10^5$  Renca tumor cells in the right flank. Tumor size was then monitored as described above.

**Cytotoxicity and cytokine assays.** At day 40 after tumor injection, mice that had experienced complete tumor regression were sacrificed to harvest the spleen. Erythrocyte-depleted splenocytes ( $5 \times 10^5$  cells/mL) were cultured in vitro with UVB-irradiated MT-901 tumor cells ( $2.5 \times 10^4$  cells/mL) in a 150 cm<sup>2</sup> flask for 5 days. On day 1, rhIL-2 was added at 120 IU/mL. On day 5, the cells were collected and dead cells were removed by density gradient. The resulting viable cells were then tested for specific cytotoxicity in a standard 4-hr <sup>51</sup>Cr-release assay, as described previously (7). Percent specific cytotoxicity was calculated as  $100 \times ([\text{experimental release} - \text{spontaneous release}] / [\text{maximal release} - \text{spontaneous release}])$ . Lytic units were then calculated as number of effector cells/ $1 \times 10^7$  cells to achieve 20% lysis ( $LU_{20}/10^7$  cells).

Aliquots of splenocytes ( $2 \times 10^6$  cells/mL) were also cultured for 48-hr in vitro with  $4 \times 10^5$  UVB-irradiated MT-901 or Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurements of murine IFN-gamma and GM-CSF release by standard ELISA (Pharmingen).

**Histologic analysis.** Tumor and the surrounding rim of normal skin and underlying connective tissue were excised at days 12, 19, and 24 after tumor injection from control and treated mice and were submitted for histologic processing. The paraffin-embedded tissues were sectioned at 4  $\mu$ m and stained with hematoxylin and eosin. Slides of sectioned tissues were prepared and evaluated by a pathologist (B.J.N.).

## Results

**Measurement of tumor apoptosis.** We first assessed the level of baseline apoptosis in a series of s.c. growing murine tumors, including chemically-induced sarcomas and a breast carcinoma. We quantitated the level of apoptosis of the dispersed solid masses by FACS analysis using the annexin V binding assay, as described (12,20,21). In our initial screening studies, a substantial proportion (32% and 41%) of two separately harvested MT-901 breast tumors represented cells undergoing early apoptosis after subcutaneous injection of an initial suspension of viable single cells (Figure 1). In contrast, the sarcomas demonstrated a relatively low baseline level of apoptosis (approximately 8%) and were resistant to treatment by DC alone administered intratumorally (data not shown). These sarcomas nonetheless were inherently weakly-immunogenic, similar to that of the MT-901 breast tumor, and likewise could elicit antitumor immunity *in vivo*, particularly when lysates were prepared and pulsed onto bone marrow-derived DC and used as the immunogen (7,16).

**Intratumoral injections of DC.** In our earlier studies (7), immunization of mice with DC alone failed to impact significantly on the growth of MT-901 mammary tumor located at either distant subcutaneous or distant pulmonary sites. In order to overcome the potential requirement for large numbers of DC to first effectively traffic to and then persist within a solid tumor mass for a sufficient period to phagocytose apoptotic cells, we examined the effect of local delivery by direct i.t. injections of bone marrow-derived DC alone. As shown in Figure 2, bone marrow-derived DC were highly efficient at engulfing whole, apoptotic MT-901 tumor cells *in vitro*. Mice with palpable, subcutaneous MT-901 mammary tumor received four courses of DC intratumorally on days 3, 10, 17, and 21 after tumor injection. As shown in Figure 3, significant tumor growth inhibition was achieved. By

day 21, tumor size in the DC-treated group averaged about 60 mm<sup>2</sup> compared to those of greater than 180 mm<sup>2</sup> in untreated, control mice ( $p < 0.01$ ). In a series of separate experiments, cohorts of mice that experienced complete tumor regression following DC treatment (with an overall cure rate of 20% based on 5 of 25 mice rendered completely disease-free) were rechallenged s.c. between 6 weeks and 10 months with a lethal dose ( $2 \times 10^5$ ) of viable MT-901 tumor cells. These mice all successfully rejected the rechallenge dose when compared to cohorts of naive, control animals (data not shown). The antitumor effect elicited by local DC administration could not be similarly achieved in control experiments that utilized normal splenocytes or allogeneic DC. As shown in Figure 4, murine bone marrow-derived DC but not splenocytes obtained from syngeneic donors resulted in substantial inhibition of the growth of MT-901 mammary tumor following intratumoral administration. By day 21, tumor size in the DC-treated group averaged about 40 mm<sup>2</sup> compared to those of greater than >120 mm<sup>2</sup> in control mice receiving equal numbers of splenocytes ( $p < 0.01$ ). Figure 5 shows that the substantial antitumor effect afforded by i.t. injections of DC was restricted to those of syngeneic but not of allogeneic origin. In other studies (not shown), allogeneic DC were shown to efficiently engulf MT-901 apoptotic tumor cells in vitro to a similar level as that of syngeneic DC. Thus, although allogeneic DC were fully capable of phagocytosis, this process was not sufficient to induce tumor regression in vivo following i.t. administration.

#### **Immunologic assessment of the antitumor effect of i.t. administered DC.**

Histologic examination of skin samples was then performed on all tumors removed following a second (day 12), third (day 19), and fourth (day 24) intratumoral administration of DC. Figure 6 depicts the results at day 24. At low and high power views, tumors from control, HBSS injected mice were extremely large with central necrosis and extensive surrounding cohesive clusters of viable malignant cells (i.e. with enlarged hyperchromatic and pleomorphic nuclei with irregular nuclear membrane and nucleoli) intermingled by PMNs, but only rare lymphocytes (Figure 6; panels A,D). At day 24, tumors removed from



total body irradiated mice injected with DC intratumorally exhibited prominent collections of large, viable malignant cells with focal areas of necrosis and PMNs, but with minimal mononuclear cell infiltration (Figure 6; panels B,E). Tumors removed from non-irradiated mice injected with DC intratumorally showed early evidence of only few viable tumor cells identified with minimal necrosis, but moderate peri-tumoral lymphocytic infiltration. Following the fourth DC injection (at day 24), only rare viable tumor cells could be identified amongst the extensive mononuclear cell infiltrate that generally had replaced the normal upper and deep dermis (Figure 6; panels C,F). Focally, small gland formation was observed amongst the MT-901 tumor cells in which surrounding lymphocytes were seen in close proximity. No such evidence of differentiation by these tumor cells was seen in any of the above two control groups.

In order to define further the nature of the host-derived lymphoid component, we selectively depleted CD8<sup>+</sup> T cells in mice by the systemic administration of specific monoclonal antibody, as described previously (7,24). Similar to our earlier findings reported with whole tumor lysate-pulsed DC immunizations, which demonstrated a predominant role of CD8<sup>+</sup> T cells (7), removal of this immune cell subset significantly reduced the capacity of DC injected intratumorally to inhibit the growth of the MT-901 mammary tumor (Figure 7). In additional studies (not shown), sublethal (500 rad) total body irradiation of mice before tumor injection and DC administration was also found to eliminate the antitumor effect of DC injections; all treated tumors continued to grow unabated similar to those in the control mice receiving HBSS alone, which corroborated our histologic findings (Figure 6).

**Enhancement of tumor apoptosis and DC antitumor effect by TNF- $\alpha$ .** We next evaluated whether increasing the level of apoptosis within the MT-901 tumor in vivo could augment the antitumor efficacy of DC administered intratumorally, particularly against a larger tumor mass. A single i.v. injection of 6  $\mu$ g TNF- $\alpha$  could increase the level of apoptosis in the MT-901 tumor to >60% of the mass (Figure 8). Mice with well-established subcutaneous MT-901 tumor were then treated on day 6 with DC alone intratumorally after

prior systemic administration of TNF- $\alpha$ . As shown in Figure 9, greater tumor growth inhibition was achieved by the combination compared to either treatment alone; fifty percent of mice receiving the combination therapy were rendered tumor-free.

**Enhancement of DC antitumor effect by foreign helper protein pulsing and IL-2 administration.** We have demonstrated that KLH, a strongly immunogenic carrier protein, could augment the efficacy of tumor lysate- or peptide-pulsed DC immunization in mediating successful immune priming against murine tumors; this effect could be further enhanced by the systemic administration of IL-2.<sup>1</sup> Figure 10 shows the results of a representative experiment; the upper and lower panels show tumor size measurements and overall survival, respectively. Intratumoral injections of DC when combined with the systemic administration of IL-2 could result in substantial MT-901 tumor growth inhibition; 60% of the treated mice underwent complete tumor regression ( $p < 0.05$ ). This antitumor effect could be enhanced further by pulsing DC with KLH prior to i.t. injection and IL-2 administration ( $p < 0.05$ ), which resulted in all treated animals experiencing complete tumor eradication and prolonged disease-free survival. All animals cured of established tumor were then challenged s.c. with  $1 \times 10^6$  viable MT-901 tumor cells (i.e. twice the dose level as that of the initial tumor challenge) in the left flank and  $5 \times 10^5$  viable Renca cells in the right flank to evaluate the level and specificity of protective immunity. All mice were fully protected against outgrowth of the MT-901 tumor but experienced progressive growth of the irrelevant Renca tumor on the contralateral side (data not shown).

Splenocytes harvested from mice that had experienced complete tumor eradication were examined for their functional reactivity following in vitro restimulation. As shown in Table 1, CTL with heightened activity against MT-901 tumor target cells could be generated from splenocytes of animals treated with KLH-pulsed DC plus IL-2 (333 LU) compared to DC plus IL-2 (17 LU) and to splenocytes from control, naive mice ( $< 1$  LU). No lysis by CTL was detected against the irrelevant, Renca tumor target (all  $< 1$  LU). The splenocytes were also examined for the production of cytokines, namely GM-CSF and IFN- $\gamma$

(Figure 11). Splenocytes isolated from MT-901 tumor cured mice treated i.t. with KLH-pulsed DC followed by IL-2 also produced greater amounts of GM-CSF (~2,000 pg) and IFN-gamma (>11,000 pg) when specifically stimulated in vitro with MT-901 tumor cells. Splenocytes isolated from MT-901 tumor-cure mice treated i.t. with unpulsed DC followed IL-2 also produced the two cytokines, but at significantly lower amounts. As a control for tumor specificity, low to negligible cytokine production was observed by stimulation of the splenocytes by the irrelevant, control Renca tumor.

## Discussion

The presence of increased DC numbers within solid tumor masses has been correlated in some studies with improved prognosis (reviewed in ref. 11). The data reported herein demonstrate that intratumoral injections of DC harvested from cultures of bone marrow cells in the presence of GM-CSF and IL-4 can mediate tumor growth inhibition. Similar to in vivo immunization studies employing antigen-pulsed DC (7), this tumor regression was dependent on host-derived CD8<sup>+</sup> T cells and was also relatively radiosensitive. In preliminary experiments, we have also noted that the tumor growth inhibition elicited by the local administration of DC alone but not splenocytes alone could elicit the regression of an established MT-901 breast tumor nodule distant (contralateral left flank) from the injected lesion, which again argues that the therapeutic efficacy of intratumoral injection of DC is immune mediated and is systemic in nature.

Syngeneic but not allogeneic DC could mediate tumor regression when delivered i.t., although both sources of DC could engulf MT-901 apoptotic tumor cells in vitro. Thus, the phagocytic activity of DC to efficiently remove apoptotic tumor cells within the mass was in itself not sufficient to reduce tumor growth in vivo. The lack of antitumor effect by allogeneic DC in our study is seemingly at odds with the published work of others (25,26). In those latter studies, fusions between tumor cells and allogeneic DC could elicit tumor

regression in vivo and could lead to the generation of MHC-restricted, tumor-specific CTL in vitro. It is conceivable that heterokaryons expressing both tumor cell- and DC-derived MHC molecules following electrofusion or chemical fusion in vitro could explain the difference in results between the latter studies and ours.

We demonstrated that KLH, a strongly immunogenic carrier protein to elicit T cell help, could enhance the antitumor effect of i.t. delivered DC when combined with the systemic administration of IL-2. These data confirm published studies of others (27) as well as our own<sup>1</sup>, which showed that KLH could augment by a CD4<sup>+</sup> T cell dependent mechanism the efficacy of tumor lysate- or peptide-pulsed DC immunization in mediating both successful immune priming toward and therapeutic rejection of tumors in vivo<sup>1</sup>. Splenocytes from mice treated i.t. with KLH-pulsed DC followed by IL-2 administration displayed heightened levels of CTL activity as well as IFN-gamma and GM-CSF secretion in a tumor-specific fashion (Table 1 and Figure 11). These findings are of particular interest because both cytolytic and noncytolytic, tumor-specific tumor-infiltrating lymphocytes have been shown to mediate potent antitumor effects in vivo upon adoptive transfer (28,29).

Recent evidence has shown that immature DC can readily acquire antigen(s) by uptake of apoptotic cells, which in turn can elicit MHC class I-restricted CTL (12,14). Such a process may play an important physiologic role in vivo in the acquisition of foreign antigens in vivo, including those derived from tumors, virally-infected and normal tissues, as well as organ transplants. Moreover, it has been shown that necrotic, but not apoptotic, cells can trigger maturation of DC in vitro (30). Thus, it is conceivable that the balance between the levels of apoptotic vs. necrotic cells within a tumor mass may influence the capacity of DC to trigger an effective immune response in situ, which may lead to a good vs. a poor prognosis, respectively. We had reported previously that bone marrow-derived DC, at a relatively immature stage, could efficiently phagocytose dextran particles (15), including those of 500,000 m.w. In this regard, we also showed in Figure 2 that DC were readily capable of efficiently engulfing intact, apoptotic MT-901 breast tumor cells.

In our current study, the MT-901 mammary tumor was found to have a prominent baseline level of cells undergoing early apoptosis within the mass. Moreover, the data of Figures 8 and 9 demonstrated that the administration of TNF- $\alpha$  could mediate increased tumor apoptosis as well as could enhance the antitumor effect elicited by the local delivery of DC. We had demonstrated previously the antitumor effects of recombinant TNF- $\alpha$  in a variety of murine tumor models when administered alone (31,32), or combined with chemotherapy (33) or IL-2 (34). We also showed that TNF- $\alpha$  mediated the antitumor effect in vivo by a combination of apoptotic, vascular, and immune T cell mechanisms (31-34).

Future studies will determine whether or not other interventions that can selectively increase tumor apoptosis in situ and/or enhance elicited host T cell immunity will result in more effective tumor regression by locally (or perhaps systemically) introduced ex vivo generated DC alone or DC generated directly in situ by the in vivo use of recombinant FLT-3L and CD40L (35). These efforts will be particularly important for tumors with relatively low apoptotic cell indices, which are also resistant to DC therapy alone. As examples, the systemic administration of a trimeric form of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to elicit apoptosis and actively suppress certain human and murine tumors in vivo without demonstrable toxic side effects to normal tissues (36-38). In addition, we reported previously that the systemic administration of IL-2 could augment the antitumor effects of tumor lysate-pulsed DC vaccines (16); thus, arguing for its use in the setting of tumor apoptosis-inducing agents and local DC administration. We have also shown previously in murine tumor models that the administration of several distinct chemotherapeutic agents (e.g., cyclophosphamide, 5FU, BCNU, DOX) can augment the antitumor efficacy of both TNF- $\alpha$  (33) and IL-2 (39). Taxol (paclitaxel) can mediate tumor apoptosis directly (40,41) and cisplatin can substantially augment the level of tumor

apoptosis induced by intratumoral injections of the adenovirus-p53 vector (42) in both murine and human tumors. Local delivery of a recombinant adenovirus vector encoding a wild-type p53 cDNA (Adv5-p53) has resulted in significant apoptosis of a variety of murine and human tumors experimentally (42-44) as well as more recently in phase I clinical trials in patients with advanced non-small-cell lung cancer (45) and recurrent head and neck squamous cell carcinoma (43). In laboratory studies, the administration of cisplatin (or VP-16) before local delivery of Adv5-p53 resulted in enhanced tumor apoptosis in vitro and in vivo as well as in enhanced antitumor effects in vivo (42).

Although we have focused on one type of DC, additional comparisons are needed. It has been suggested that the state of maturation of DC may be important for their optimal use in immunization strategies (46,47). Strategies that have resulted in DC maturation include the use of CD40L (35, 48-50), LPS (50), monocyte conditioned medium (51), and, in our own published work and that of others, TNF- $\alpha$  (52,53). Also of importance to the use of DC in our models is the discovery of DC subsets or subpopulations, which differ in their capacity to elicit antigen-specific Th1/Tc1 vs. Th2/Tc2 immune responses (54,55). In this regard, distinct roles of antigen-specific Th1/Tc1 and Th2/Tc2 cells may predominate during eradication of established murine tumors in vivo (56,57). Moreover, it remains to be determined if site directed injections of immature DC alone into apoptotic tumor-involved lymph nodes will lead to a more efficient means of eliciting both a local and systemic immune response compared to that with tumor lysate or peptide(s)-pulsed DC injected into uninvolved lymph nodes (9).

#### Acknowledgment

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**Table 1. CTL activity of splenocytes from mice experiencing tumor eradication by intratumoral DC**

Treatment*	Target cell lysis	
	MT-901	Renca
KLH-pulsed DC	333	<1
DC	33	<1
None	<1	<1

Values are based on  $LU_{20}/10^7$  cells as measured in a standard 4-hr  $^{51}\text{Cr}$ -release assay.

\*Spleens were harvested from BALB/c mice that had experienced MT-901 tumor eradication following i.t injections of DC (see Materials and Methods for details). Splenocytes ( $5 \times 10^5/\text{mL}$ ) were cultured with irradiated MT-901 tumor cells (20:1 ratio) for 5 days. At day 1, IL-2 was added at 120 IU/mL. Cells were harvested at day 5 for testing of CTL activity.

## Figure legends

Figure 1. Detection of cells undergoing apoptosis within 8-day subcutaneous MT-901 tumors. Tumor cell suspensions were made and analyzed by FACS for annexin V-FITC (ANN) and propidium iodide (PI) stained cells as described in Materials and Methods. The two histograms shown represent tumors obtained from two separate mice. The upper right hand quadrant represents ANN<sup>+</sup>/PI<sup>+</sup> and the lower right hand quadrant represents ANN<sup>+</sup>/PI<sup>-</sup> staining cells; the latter is indicative of cells in the early phases of apoptosis.

Figure 2. Bone marrow-derived DC efficiently engulf apoptotic MT-901 breast tumor cells. Transmission electron microscopy demonstrates the presence of an intact apoptotic tumor cell within the dendritic cell at 15 hr. Magnification 9375x.

Figure 3. Direct intratumoral injections of bone marrow-derived DC inhibit the growth of established subcutaneous MT-901 mammary tumor. BALB/c mice received  $3 \times 10^6$  viable MT-901 tumor cells subcutaneously on day 0. The mice were injected on days 3, 10, 17, and 21 with  $1 \times 10^6$  DC into the tumor. A control group received HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters. Data are reported as the average tumor area  $\pm$  SEM of 5 mice per group.

Figure 4. Direct intratumoral injections of DC but not splenocytes inhibit the growth of established subcutaneous MT-901 mammary tumor. On day 0, BALB/c mice received  $3 \times 10^6$  viable MT-901 tumor cells subcutaneously. Mice were then injected on days 3, 10, 17, and 21 with  $1 \times 10^6$  DC into the tumor. Control groups received naive splenocytes alone or



HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters. Data are reported as the average tumor area  $\pm$  SEM of 5 mice per group.

Figure 5. Direct intratumoral injection of syngeneic DC but not allogeneic DC inhibit the growth of established MT-901 mammary tumor. DC were administered beginning on day 14 after s.c. tumor injection. Allogeneic DC were generated from bone marrow cells of C57BL/6 mice, as described in Materials and Methods. The size of the tumors was recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters. Data are reported as the average tumor area  $\pm$  SEM of 10-11 mice per group.

Figure 6. Histologic analysis of tissue samples removed from the skin of mice injected with MT-901 mammary tumor cells and either: HBSS (panels A,D), DC following host total body irradiation (panels B,E), or DC alone (panels C,F). Marked host-derived mononuclear cell infiltrates are seen for the latter group. Photographs represent samples taken at day 24; mice in the DC-treated groups received four intratumoral injections of  $1 \times 10^6$  DC.

Figure 7. Host-derived CD8<sup>+</sup> T cells participate in the in vivo antitumor effect of intratumoral injections of DC. BALB/c mice were depleted of CD8<sup>+</sup> T cells or received rat IgG for isotype control of antibody function. On day 0, all mice received  $3 \times 10^6$  viable MT-901 tumor cells subcutaneously. Mice were then injected on days 3, 10, 17, and 21 with  $1 \times 10^6$  DC into the tumor. Control mice received HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters. Data are reported as the average tumor area  $\pm$  SEM of 5 mice per group.

Figure 8. Systemic administration of TNF- $\alpha$  increases the level of early apoptosis of the

MT-901 breast tumor mass. BALB/c mice were injected with a single dose of 6  $\mu$ g TNF- $\alpha$  (right panel) or HBSS (left panel) intravenously. Twenty four hours later, tumor cell suspensions were made and analyzed by FACS for annexin V-FITC (ANN) and propidium iodide (PI) stained cells as described in Materials and Methods. The upper right hand quadrant represents ANN<sup>+</sup>/PI<sup>+</sup> and the lower right hand quadrant represents ANN<sup>+</sup>/PI<sup>-</sup> staining cells; the latter is indicative of cells in the early phases of apoptosis.

Figure 9. Systemic administration of TNF- $\alpha$  enhances the antitumor effect of intratumoral injections of DC in vivo. BALB/c mice with day 6 subcutaneous tumor received DC ( $1 \times 10^6$ ) intratumorally on days 6, 14, and 20 in combination with intravenous TNF- $\alpha$  on days 5 and 13. Control mice received DC, TNF- $\alpha$ , or HBSS alone. Tumor size was assessed twice weekly and recorded as tumor area (in mm<sup>2</sup>) by measuring the largest perpendicular diameters with calipers. Data are reported as the average tumor area  $\pm$  SEM.

Figure 10. KLH and IL-2 enhances the therapeutic efficacy of DC delivered intratumorally, Normal BALB/c mice received  $5 \times 10^5$  viable MT-901 tumor cells s.c. on day 0. The mice were then injected on days 7, 10, 13, 17 and 20 with  $2 \times 10^6$  DC in a 50  $\mu$ L i.t. injection. Control groups of mice received either unpulsed DC ( $2 \times 10^6/50 \mu$ L), normal splenocytes ( $2 \times 10^6/50 \mu$ L), or HBSS alone. IL-2 was given intraperitoneally (i.p.) twice daily at 60,000 IU in 0.5 mL HBSS for 2 days after each treatment. The size of the tumor was assessed (upper panel) and survival was followed as recorded as the percentage of surviving animals over time (in days) (lower panel).

Figure 11. Intratumoral delivery of KLH-pulsed DC plus the systemic administration of IL-2 enhance specific T cell production of cytokines. Mice were treated as described in Materials and Methods. Following tumor eradication, aliquots of splenocytes ( $2 \times 10^6$  cells/mL) were cultured for 48-hr in vitro with  $4 \times 10^5$  UVB-irradiated MT-901 or

irrelevant Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurements of murine GM-CSF (upper panel) and IFN-gamma (lower panel) release by standard ELISA (in pg/mL; mean  $\pm$  SEM of triplicate samples).

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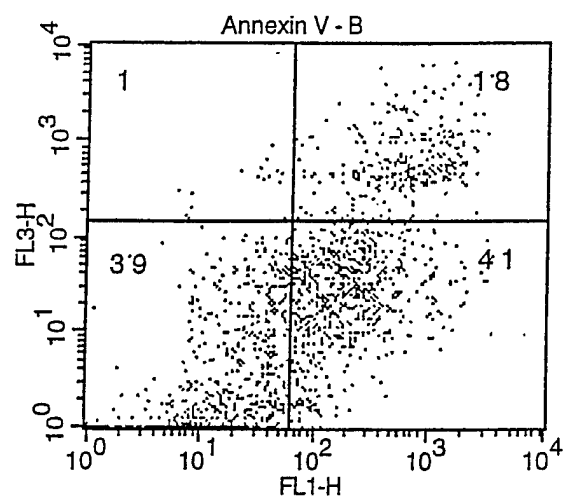
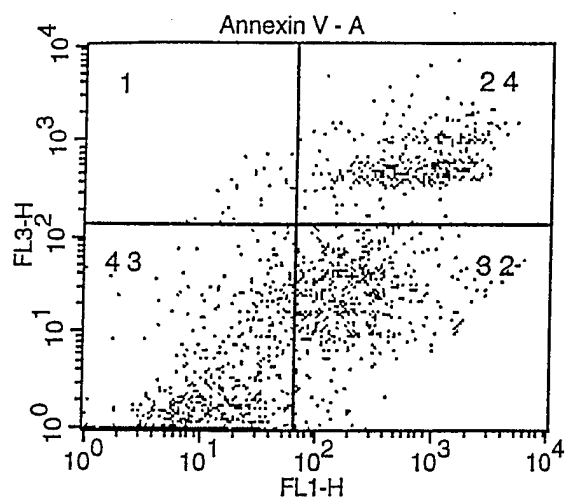
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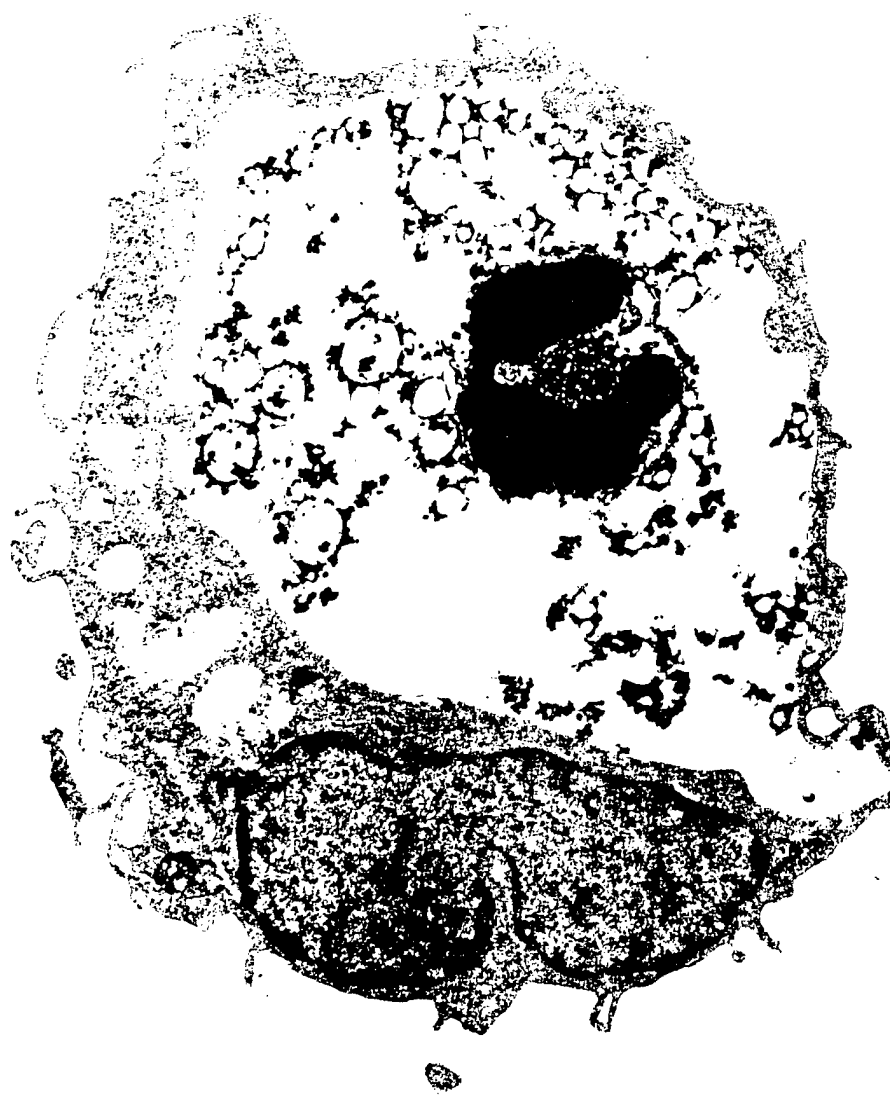
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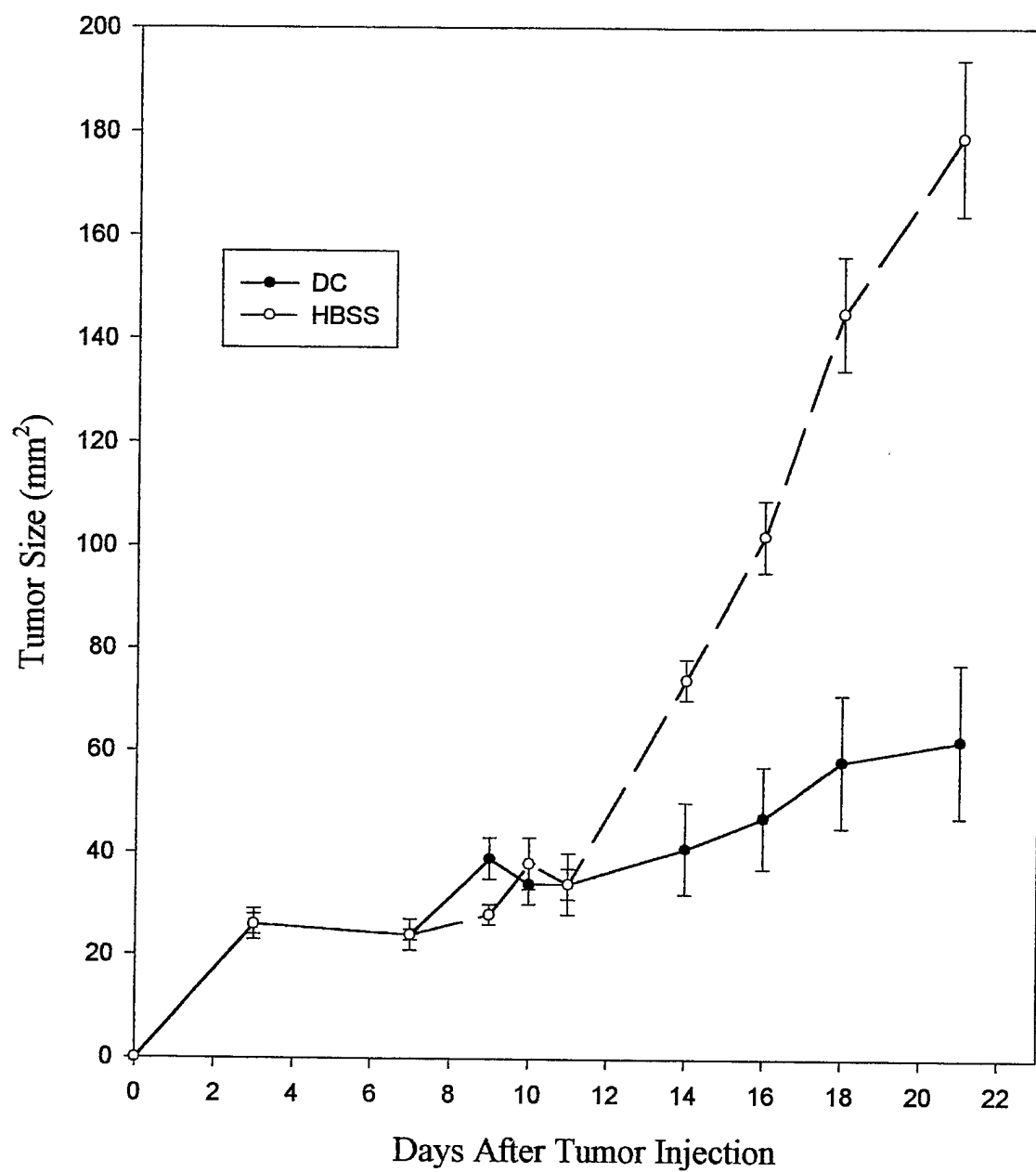
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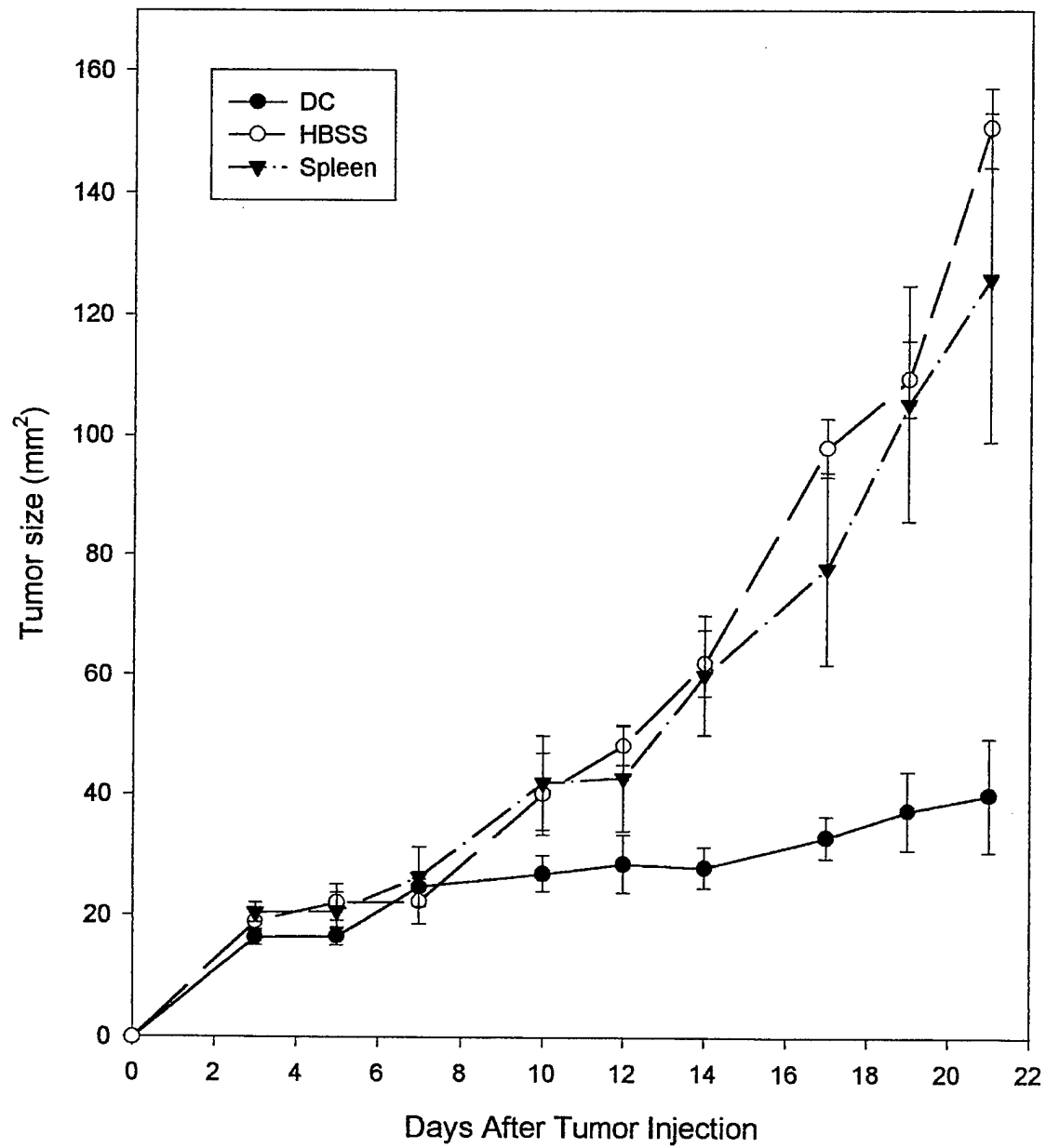


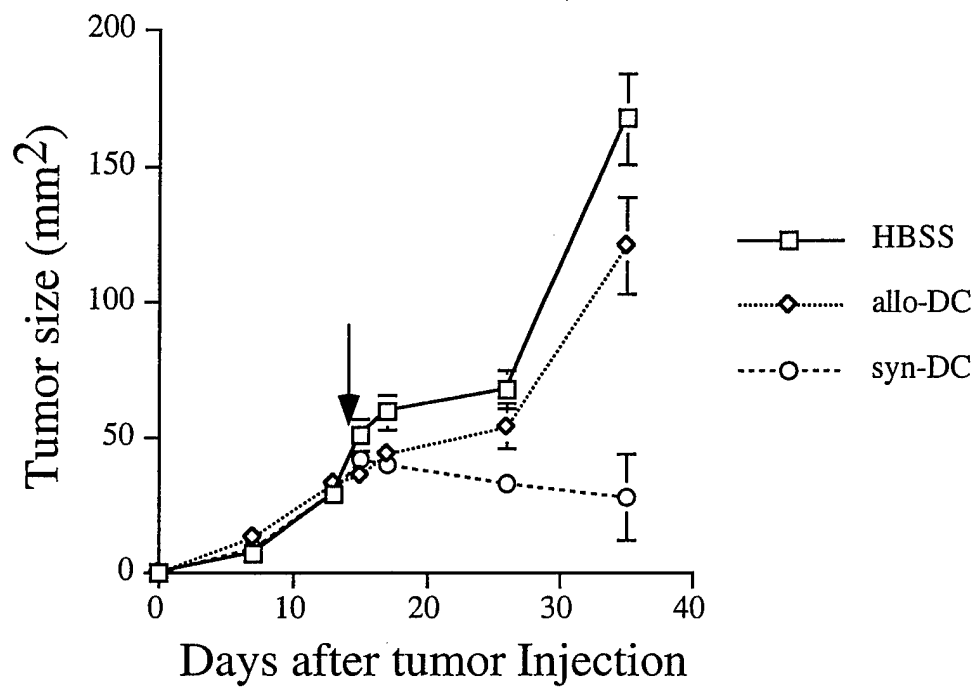
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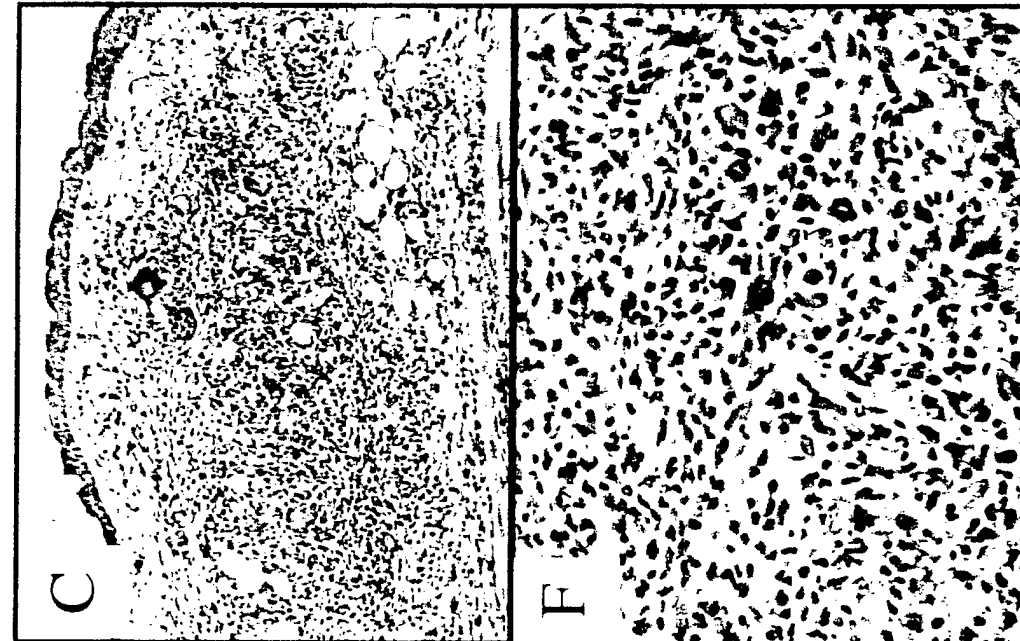
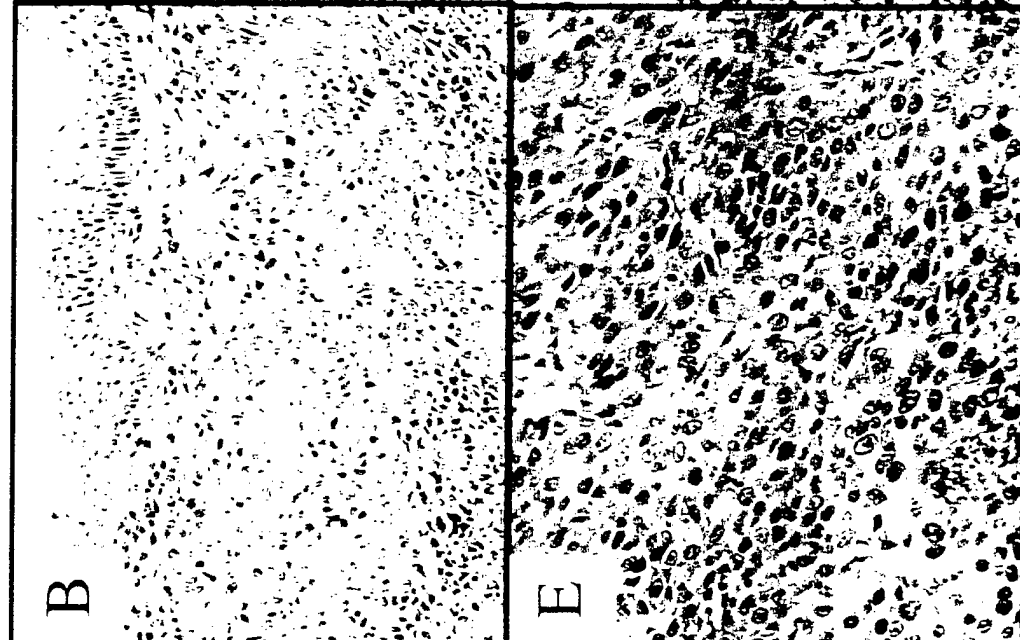
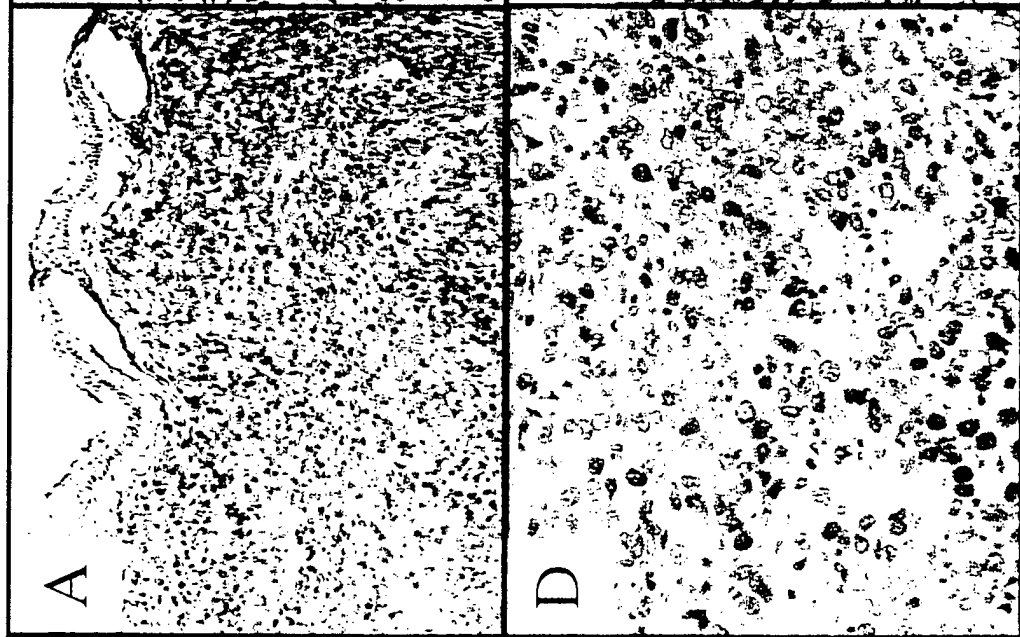


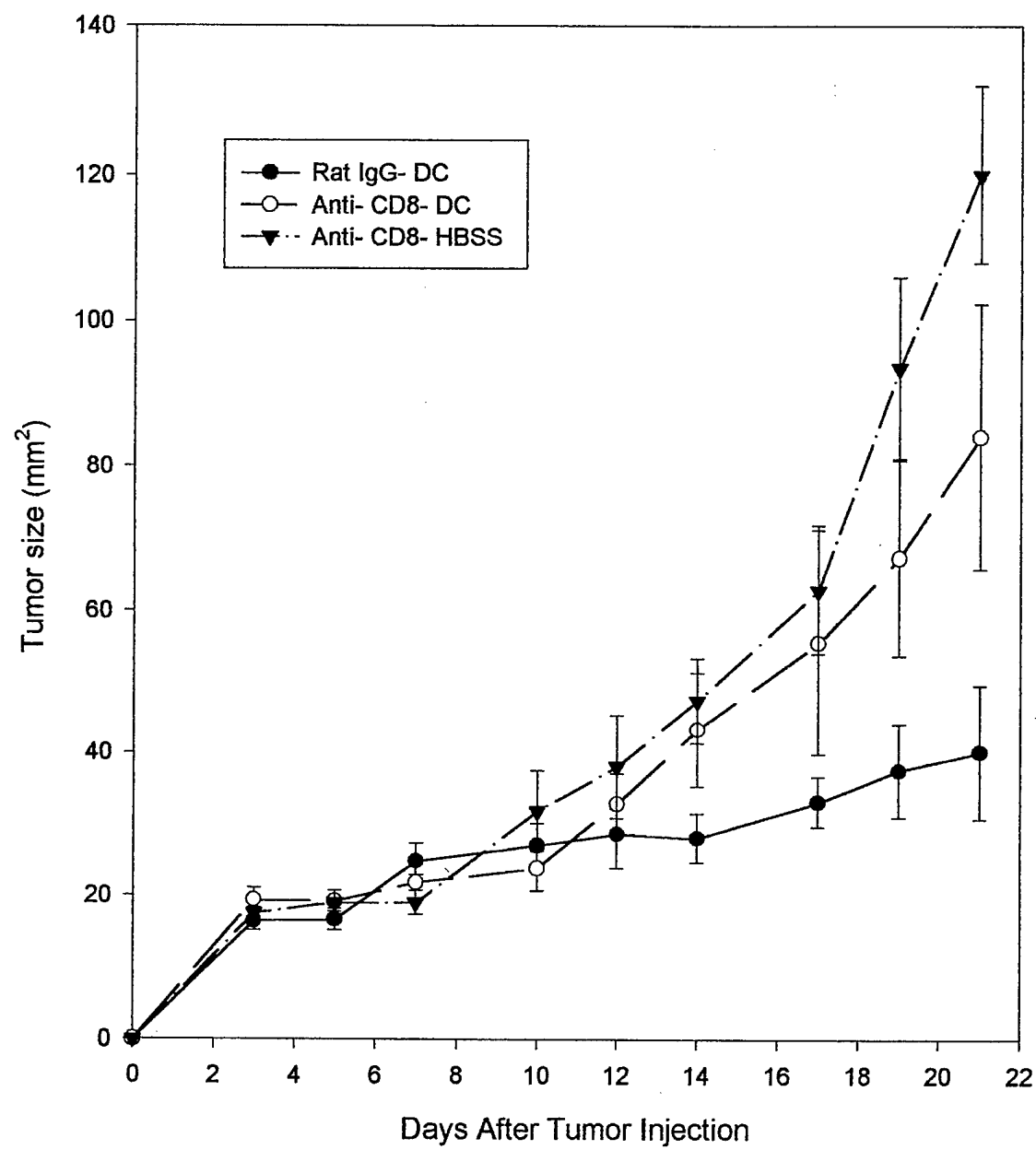






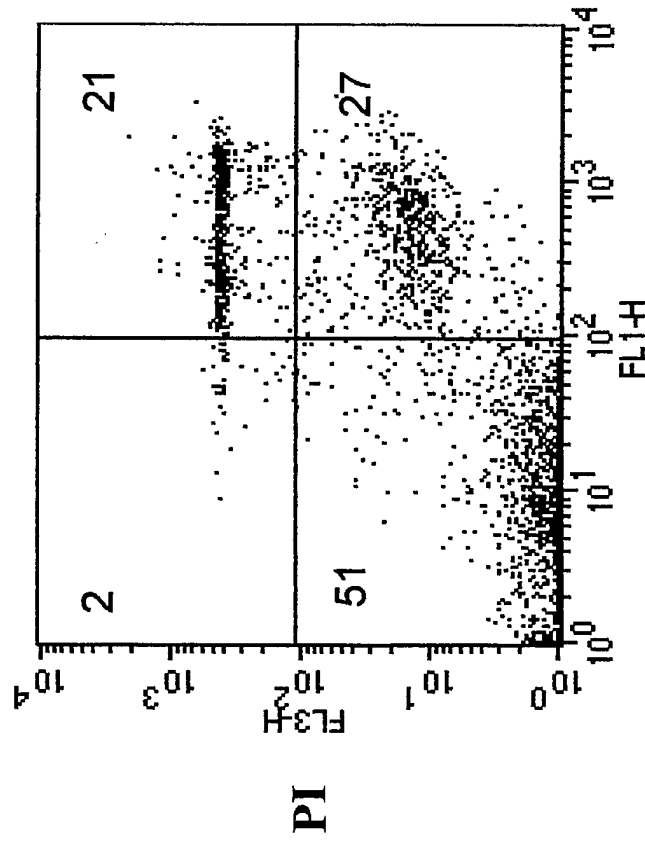




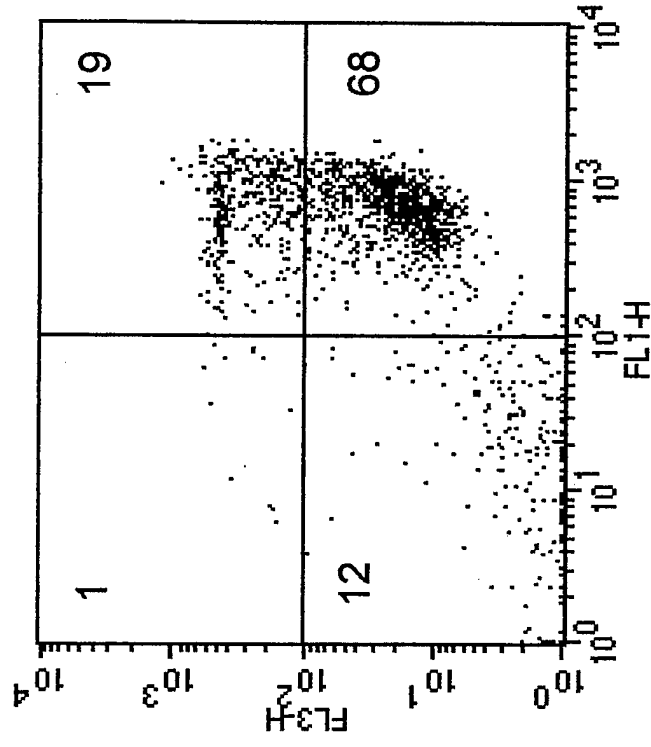




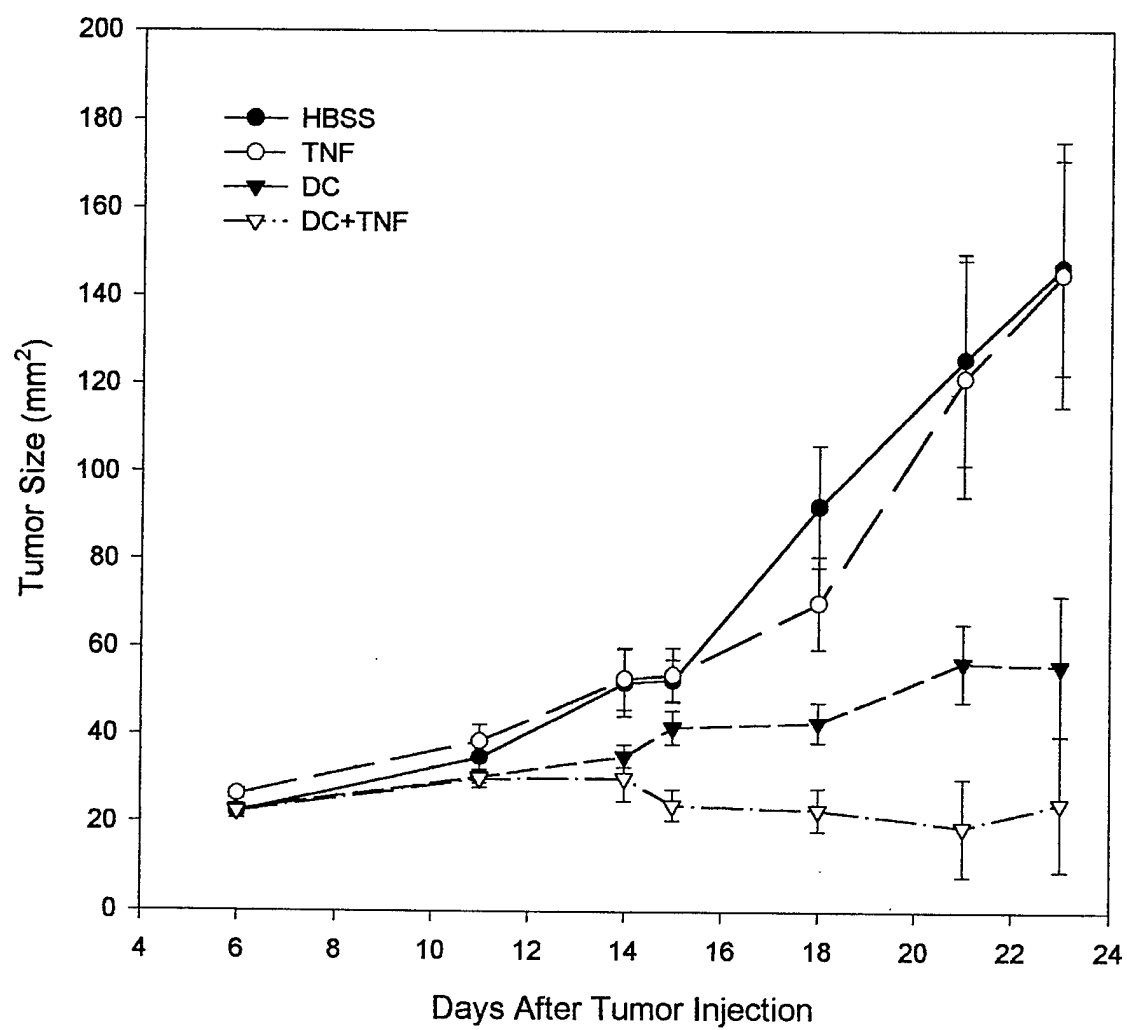
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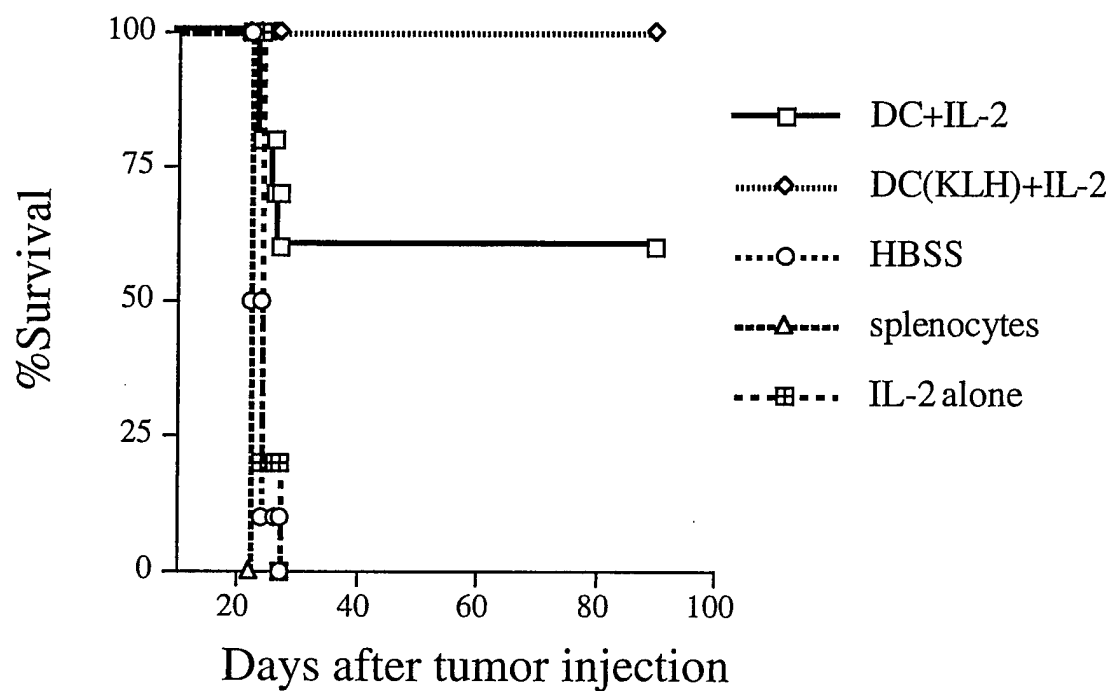
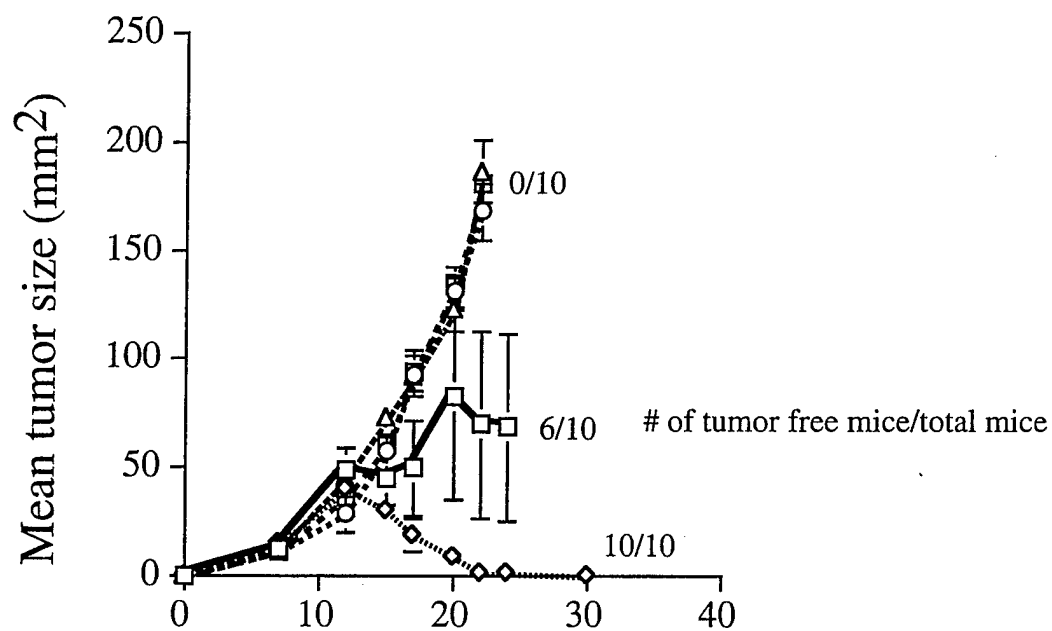


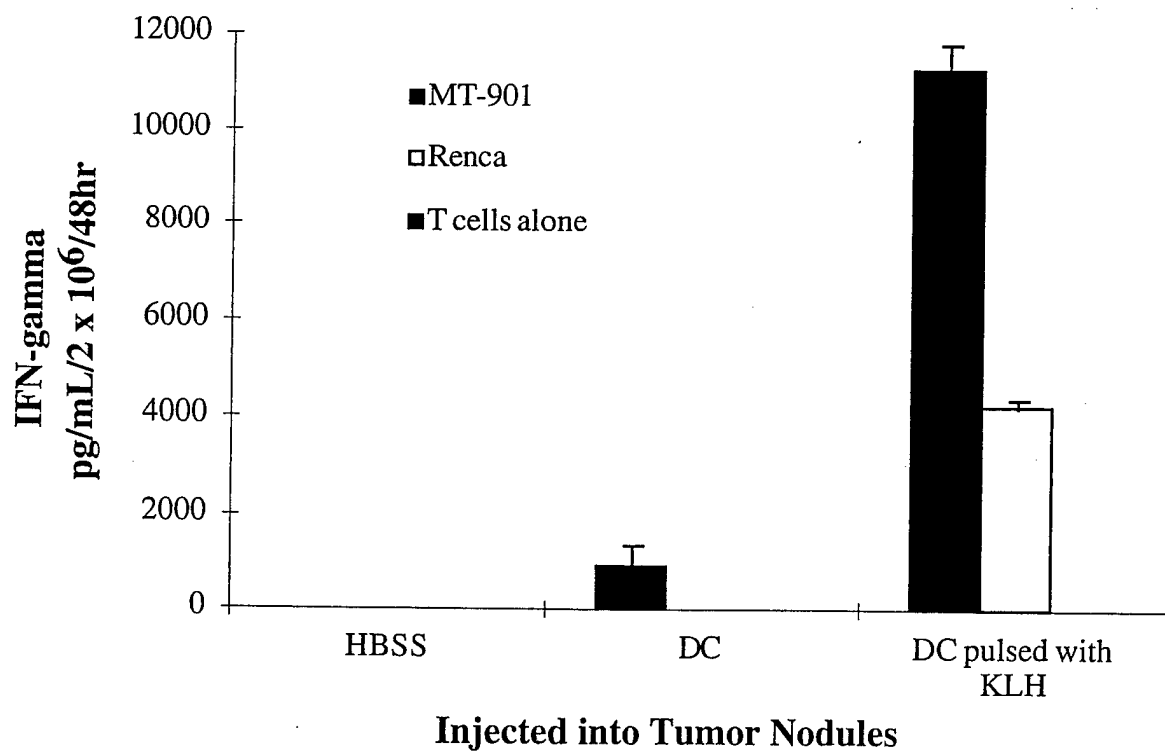
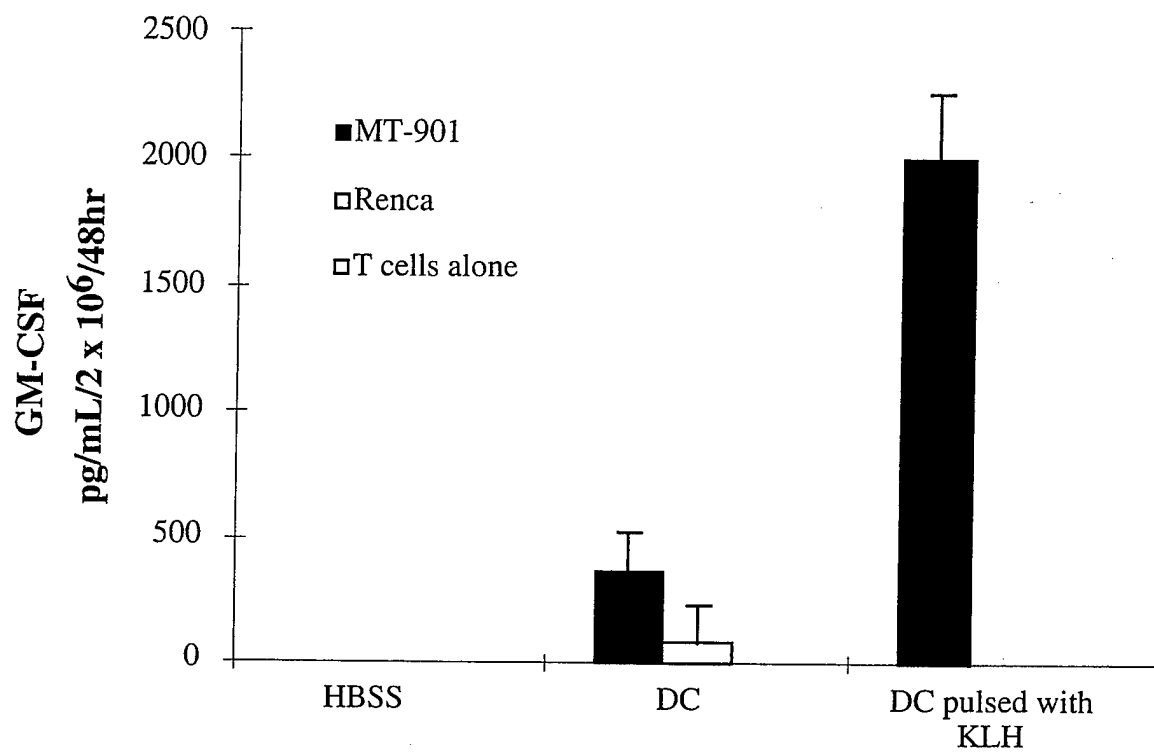
TNF- $\alpha$



Ann V







# **T-Cell Dependent Antitumor Immunity Mediated by Secondary Lymphoid Tissue**

## **Chemokine (SLC): Augmentation of Dendritic Cell Based Immunotherapy**

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Running Title: Anti-tumor immunity elicited by Secondary Lymphoid Tissue Chemokine

Key words: Secondary Lymphoid Chemokine (SLC), dendritic cells, melanoma, immunotherapy, gene therapy

## **Abstract**

Secondary lymphoid tissue chemokine (SLC) is a CC chemokine that is selective in its recruitment of naïve T-cells and dendritic cells (DC). In the lymph node, SLC is believed to play an important role in the initiation of an immune response by co-localizing naïve T-cells with DC presenting antigen. Here, we employed SLC as a treatment for tumors established from the poorly-immunogenic B16 melanoma. Intratumoral injections of SLC inhibited tumor growth in a CD8+ T-cell dependent manner. SLC elicited a substantial infiltration of DC and T-cells into the tumor, coincident with the anti-tumor response. We next utilized SLC gene-modified DC as a treatment of established tumors. Intratumoral injections of SLC expressing DC resulted in tumor growth inhibition that was significantly better than either control DC or SLC alone. Distal site immunization of tumor-bearing mice with SLC gene-modified DC pulsed with tumor lysate elicited an anti-tumor response while control DC did not. We also found that s.c. injection of lysate-pulsed DC expressing SLC promoted the migration of T-cells to the immunization site. This report demonstrates that SLC can both induce anti-tumor responses and enhance the anti-tumor immunity elicited by DC.

## Introduction

In order to induce an immune response against an established tumor, T-cells specific for antigens expressed by the tumor (TAA<sup>1</sup>) must become activated, most likely by specialized cells presenting those antigens (APC) (1). However, since most of the known TAA are non-mutated self proteins, the T-cells that can mediate tumor eradication are i) self reactive by definition and ii) probably exposed to those antigens in the periphery long before any active immunotherapy is initiated (2). Therefore, the goal of a therapeutic cancer vaccine is the proper uptake and presentation of TAA by APC such that a specific anti-tumor response is initiated. Considerable effort has been made in the study of antigen types (e.g. peptides, irradiated tumor cells, gene fragments), adjuvants (e.g. chemical adjuvants, cytokines, dendritic cells) and modes of delivery (e.g. DNA, pulsed dendritic cells, peptide/adjuvant complexes) with the explicit intent of optimizing the priming of TAA specific T-cells (3).

Due to their potent ability to stimulate T-cells, particularly naïve T-cells, DC are generally conceived as the most potent members of the class of APC (4). Based in part on current protocols which enable the generation of large numbers of DC from peripheral blood (5;6), DC have been proposed as the basis of cancer vaccines. Indeed, encouraging results from

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<sup>1</sup> Abbreviations used in this paper: SLC, secondary lymphoid tissue chemokine; GFP, green fluorescent protein; DC, dendritic cells; APC, antigen presenting cells; TAA, tumor associated antigens

preclinical and clinical studies highlight the promise of DC based cancer immunotherapy (7). However, in these early studies, complete regression of tumors is not seen in the majority of patients, suggesting that modification of DC based vaccines is required before they become a widespread treatment modality (8-10).

Genetic modification of DC to express either tumor antigens or immunomodulatory proteins has met with success in preclinical animal models of tumor treatment (11;12). Conceivably, DC that process and present TAA as transgene products present those antigens for a longer time in vivo than ex-vivo pulsed DC, due to continuous gene expression and MHC loading. DC that express cytokines may represent a longer-lived or more immunostimulatory DC depending upon the type of cytokine gene expressed. DC genetically modified to express GM-CSF or Lymphotoxin (lptn, a C chemokine), and pulsed with antigens induce a stronger anti-tumor response than control gene-modified DC (13-15). In another treatment model, in which DC are injected unpulsed directly into the tumor, expression of IL-12 (16) or IL-7 (17) by the DC improves their therapeutic efficacy.

It is becoming increasingly more evident that chemokines play an integral role in the initiation of a specific immune response (18). Chemokines are a family of small secreted molecules that mediate leukocyte migration (19). One such chemokine, secondary lymphoid tissue chemokine (SLC) is a CC chemokine found on high endothelial venules and within the T-cell zones of both spleen and lymph nodes (20-23). SLC is capable of recruiting both dendritic cells (DC) and naïve T-cells via the CCR7 receptor found on both cell types (24-27). Because of its expression pattern and that of its receptor, SLC has been postulated to play an important role in the priming of naïve T-cells by DC (28). Indeed, mice deficient in either SLC or CCR7 have



lower steady state levels of T-cells in peripheral lymph nodes, reduced migration of hapten primed DC to draining nodes and impaired immune responses to encountered antigens (29;30).

Because both DC and naïve T-cells express CCR7, the ligand for SLC, we hypothesized that SLC could be used to initiate or enhance anti-tumor immunity in mice bearing established tumors. We utilized a mouse model of a poorly immunogenic B16-BL6 melanoma to determine the effects of SLC on the initiation of an anti-tumor response. We used three distinct treatment models to assess the therapeutic efficacy of SLC: 1) direct intratumoral injections of recombinant SLC; 2) intratumoral injections of DC genetically-modified to express SLC and 3) distal site immunizations of SLC expressing DC that were pulsed with whole tumor lysate (31;32). We utilized an adenovirus vector encoding SLC to modify DC to express high levels of this chemokine. Our results show that SLC can induce a strong anti-tumor response that results in significant infiltration of immune effector cells into treated tumors and that genetic modification of DC to express SLC enhances their capacity to elicit tumor rejection *in vivo*.

## Materials and Methods

**Animals.** C57BL/6J (denoted B6) and BALB/c female mice (six to 8-week old) were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Animal Maintenance Facility at the University of Michigan Medical Center for at least one week prior to use. Animals were 8 to 12 weeks of age before use in studies

**Medium and Cytokines/Chemokines.** Complete Medium (CM) consisted of RPMI medium 1640 with 10% heat-inactivated fetal calf serum (FCS), 0.1 mM nonessential amino acids, 1  $\mu$ M sodium pyruvate, 2 mM fresh L-glutamine, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, 50  $\mu$ g/ml gentamicin, 0.5  $\mu$ g/ml fungizone and  $5 \times 10^{-5}$  M 2-ME. Recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF; specific activity:  $\geq 5 \times 10^6$  U/mg) was obtained from Immunex Corp. (Seattle, WA); recombinant murine interleukin 4 (IL-4;  $2.8 \times 10^8$  units/mg) was obtained from Schering-Plough Pharmaceutical Research Institute (Kenilworth, NJ); recombinant murine SLC was obtained from Chiron Corp. (Emeryville, CA); recombinant murine RANTES was purchased from R & D Systems (Minneapolis, MN)

**Tumor Cell lines.** B16-BL6 is derived from B6 mice and is a poorly immunogenic melanoma of spontaneous origin (33). MT-901 is a subline of the MT-7 tumor cell line derived from a dimethylbenzanthrene-induced mammary carcinoma in BALB/c mice (34). Tumors were cultured in vitro in CM and were used before the 10<sup>th</sup> passage.

**Microchemotaxis.** Splenocyte responder cells were generated by gently rubbing spleens between frosted glass slides and passing over a nylon mesh filter (70  $\mu\text{m}$ ). Red blood cells were lysed and the splenocytes resuspended in RPMI-1640 containing 5% FCS (RPMI-FCS) and subjected to two rounds of adherence to plastic at 37°C. Nonadherent cells were resuspended to  $1 \times 10^7$  cells/ml in RPMI-FCS prior to use in microchemotaxis assays. DC responders were obtained from 7 day bone marrow cultures as described below and were used at  $2.5 \times 10^6$  cells/ml in RPMI-FCS. Assays were performed in 24 well plate format with 6.5 mm diameter, 5  $\mu\text{m}$  pore polycarbonate transwell insets (Costar, Cambridge, MA) in duplicate samples. SLC was added to the lower chambers at the indicated concentrations in a volume of 600  $\mu\text{l}$  and incubated at 37°C for 30 minutes prior to addition of cells. 100  $\mu\text{l}$  of cell suspension were added to the top chamber and the assay was carried out at 37°C in a humidified incubator with 5%  $\text{CO}_2$ . A 1:5 dilution of the cells was also directly added to the lower chamber of 2 wells for determination of the input amount. After 2 hours the assay was stopped by the removal of the inserts followed by the addition of  $10^4$  polystyrene beads (15  $\mu\text{m}$  diameter; Bangs Laboratories, Fishers, IN) to the lower chamber. Samples were stained with antibodies against CD4 and CD8 (splenocytes) or MHC II and CD86 (DC) and counted on a FACScaliber (Becton Dickinson, San Jose, CA). In separate experiments, CD4 and CD8 cells were counterstained for expression of CD62L (all antibodies from Pharmingen, San Diego, CA). The number of cells in each sample (and the input) was determined by the equation: (# of cells events/# beads events)  $\times 10^4$  beads/sample. The

percent migration in each sample (% input) was determined by the equation:  $[\# \text{ of cells in sample} / (\# \text{ of cells in input} \times 5)] \times 100$ .

**Treatment of Established Tumors with SLC.** B6 and BALB/c mice were injected s.c. with  $1 - 3 \times 10^5$  B16 or  $1 \times 10^6$  MT901 cells (> 95% viability) respectively in the right flank. On day 6 (B16-BL6) or day 7 (MT901) when tumors were palpable, intratumoral injections of SLC (3  $\mu$ g per dose, unless otherwise specified) in 50  $\mu$ l of PBS + 0.05% normal mouse serum were initiated. Control groups received vehicle alone or s.c. injections of SLC in the left flank. In some experiments, B6 mice were depleted of CD4+ or CD8+ T-cells by i.p. injections of 200  $\mu$ l anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43) mAbs (both from ATCC) 4 and 3 days before receiving the first treatment of SLC and 3, 7 and 10 days after treatment began. Control mice received isotype IgG2b (Sigma, St. Louis, MO). T-cell subset depletion was checked by FACS analysis and was determined to be completely effective and selective (data not shown). Tumor size was monitored twice weekly and recorded as tumor area (in  $\text{mm}^2$ ) by measuring the largest perpendicular diameters with vernier calipers. Data are reported as the average tumor area  $\pm$  S.E.M.; five or more mice per group.

**Generation of Bone Marrow-Derived DC.** Erythrocyte depleted bone marrow cells flushed from the femurs and tibias of B6 mice were cultured in 10 ng/ml GM-CSF and 10 ng/ml IL-4 at  $1 \times 10^6$  cells/ml in CM. At day 3 fresh cytokines were added and non-adherent cells were harvest on days 4 – 7 by gentle pipetting. DC were enriched by density centrifugation over 14.5% (w/v) matrizamide (Sigma, St. Louis, MO) (35). The low density population (bufficoat) was washed

several times in RPMI-1640 + 2% FCS prior to use. The resulting DC population was > 80% positive for co-expression of MHC II, CD11c, CD40, CD80 and CD86 (data not shown).

**Tumor Harvest for Immunohistochemistry and FACS analysis.** B6 mice received  $2 \times 10^5$  B16-BL6 cells s.c. in the right flank and were treated with daily intratumoral injections of 3  $\mu$ g SLC (or vehicle as control) from days 6 – 10. For immunohistochemical analysis of DC, tumors were harvested and snap frozen in liquid N<sub>2</sub>, and sections analyzed by one of us (B.J.N.) for the presence of DC with the DEC-205 specific antibody (Serotec, Raleigh, NC). DC were counted in 10 high powered fields (40X) per section (2 sections per tumor) in a blinded fashion. For analysis of T-cell infiltration B16-BL6 tumors were measured, harvested, removed of extraneous tissue and digested for 2 hours at room temperature in 10 mg/ml Type IV collagenase (Sigma, St. Louis, MO) with constant stirring. Digested tumors were passed over a 70  $\mu$ m nylon mesh, washed once with HBSS and resuspended in PBS + 3% BSA to  $\sim 1 \times 10^6$  cells/ml. Polystyrene beads (15  $\mu$ m diameter) were added to the samples to achieve a concentration of  $5 \times 10^5$  beads/ml. Samples were stained for the presence of CD4 and CD8 with phycoerythrin (PE) conjugated antibodies (Pharmingen). Samples were analyzed by FACS with counting of 50,000 lymphocyte sized events (based on splenocyte controls). The number of infiltrating CD4 or CD8 cells per tumor was determined by the following equation: (# of PE events/# beads events)  $\times 5 \times 10^5 \times$  cell sample volume. Because the tumors were of different sizes, the data was normalized to the tumor volume by dividing the total number of infiltrating CD4+ (or CD8+) cells by the tumor volume using the volume equation  $V$  (in mm<sup>3</sup>) =  $0.4(ab^2)$ , where  $a$  is the long diameter and  $b$  is the short diameter.

**Preparation of adenoviral vectors.** aD2028#16 (Ad-SLC) carries an SLC expression cassette in its E1 region. The cassette was excised as an SfiI-BspLU11I fragment from pCMVII-Amp-SLC (Chiron), blunt-ended, and cloned into the BglII site of shuttle vector pD1954-BglIII. The resulting plasmid contains Ad DNA from 0-1, 9.3-20.2, and 98.2-100 map units. This plasmid was digested with BspEI to separate the left and right ends of the Ad genome and recombined in BJ5183 cells (36) with Hirt prep DNA (37) prepared from mammalian cells infected with an E1-, E3-deleted adenovirus. The intact Ad-SLC genome was released from the resulting plasmid (pD2028#16) by restriction digest and transfected into C7 cells to recover virus (38;39). pAdEasy1-GFP, containing the Ad-GFP genome, was a gift from Dr. Bert Vogelstein (40). Viruses were propagated on C7-cell monolayers and purified on CsCl gradients according to a standard protocol (41). Purified virus was dialyzed against 20 mM HEPES (pH 7.4) containing 5% sucrose, aliquoted, and frozen in a dry ice/ethanol bath (42).  $OD_{260}$  was determined after particle disruption at 56°C for 10 minutes in 0.1% SDS, 10 mM Tris-Cl (pH 7.4), and 1mM EDTA. Particle concentration was calculated using an extinction coefficient of  $9.09 \times 10^{13}$  OD/ml/cm/virion (43). Plaque assays were also performed and yielded similar vector particle to infectious unit ratios for all preparations (mean  $84 \pm 11$ ).

**Genetic Modification of DC with adenoviral vectors.** DC were resuspended at a concentration of  $1 \times 10^7$  cells/ml in RPMI 1640 + 2% FCS and placed in a 15-ml conical tube. Virus was added at a ratio of 16,048 vector particles per DC, the suspension was mixed well, and the tube was incubated at 37°C for two to four hours. Nine volumes of complete medium with

10 ng/ml GM-CSF and 10 ng/ml IL-4 were then added and the cells were transferred to tissue culture dishes. Cells were incubated for 18 hr at 37°C, supernatants recovered and the cells purified by incubation in PBS with 3 mM EDTA and gentle scraping. Using an adenovirus encoding green fluorescent protein (GFP), we determined a transfection efficiency of ~40% (data not shown). In some cases the cells were cultured for 72 hours with supernatant harvest every 24 hours. The cells were washed several times in Hank's balanced salt solution (HBSS), resuspended to  $5 \times 10^6$  cells/ml and irradiated with 2,000 rad prior to use.

**Tumor Lysate pulsing of gene-modified DC.** Following adenovirus infection, DC were resuspended to  $1 \times 10^6$  cells/ml in CM containing lysate from B16-BL6 cells that had been lysed by 3 rapid freeze/thaw reactions and spun at  $\sim 100 \times g$  to remove cellular debris. The DC were pulsed at a 3:1 tumor cell to DC ratio for 18 hours (31;32). Following pulsing, the DC were collected, their cultured supernatants harvested for microchemotaxis, washed several times in HBSS, resuspended to  $5 \times 10^6$  cells/ml and irradiated with 2,000 rad prior to use.

**Quantitation of SLC production by gene-modified DC.** Since there are no currently available monoclonal antibody pairs against SLC suitable for ELISA, a microchemotaxis-based bioassay was performed to determine the amount of functional protein produced by gene-modified DC. Supernatants from DC infected with either Ad-GFP or Ad-SLC were added to the bottom chamber of 24 well plates in duplicate to quadruplicate samples (in some cases a 1:2 dilution was used) and a microchemotaxis assay with splenocyte responder cells was performed as described above. Concurrently, known amounts (10, 100, 500, 1000 and 5000 ng/ml) of recombinant SLC

were also added to separate wells in duplicate to generate a standard curve of SLC activity. The equation of the standard curve was generated by non-linear regression using GraphPad Prism software. We chose a one site binding equation  $Y = (B_{\max} \times X)/(K_d + X)$  where  $Y = \% \text{ input}$ ,  $B_{\max}$  = maximum migration,  $K_d$  = chemokine concentration for half maximal migration and  $X =$  chemokine concentration. Chemokine amounts presented as  $\text{ng}/1 \times 10^6$  cells in 18 or 24 hours were determined from the equation derived from the standard curve for each microchemotaxis assay. The  $R^2$  for each standard curve in 9 of 9 experiments was  $\geq 0.92$ .

**Treatment of established B16-BL6 tumors with gene-modified DC.** B6 mice were injected s.c. with  $5 \times 10^4$  B16 cells in the right flank. Treatment began on day 6 when palpable tumors of  $\geq 9 \text{ mm}^2$  were present. DC ( $5 \times 10^5$ ) were injected into tumors on days 6, 9 and 13. A cohort of mice were treated with daily intratumoral injections of recombinant SLC on days 6 – 10. As described above, tumor size was monitored twice weekly and recorded as tumor area (in  $\text{mm}^2$ ) by measuring the largest perpendicular diameters with vernier calipers. Data are reported as the average tumor area  $\pm$  S.E.M.; five or more mice per group.

**Analysis of T-cell migration in vivo.** B6 mice were injected intradermally with  $1 \times 10^6$  gene-modified DC that had been pulsed with B16-BL6 tumor lysate. Skin biopsies ( $1.5 \times 1.5 \text{ cm}$ ) including and surrounding the injection site were harvested 3 days after injection. The tissue was minced and digested for 2 hours at room temperature in HBSS plus 10 mg/ml collagenase (type IV), 1,500 units/ml DNase I (type IV) and 1 mg/ml hyaluronidase (type V; all from Sigma, St. Louis, MO) with constant agitation. Samples were passed through nylon mesh to remove



particulate matter and resuspended to  $\sim 1 \times 10^6$  cells/ml. Polystyrene beads were added to achieve a final concentration of  $5 \times 10^5$  beads/ml. Samples were stained for the presence of T-lymphocytes using PE conjugated antibodies against CD4 and CD8. The number of infiltrating CD4 or CD8 cells per tumor was determined by the following equation: (# of PE events/# beads events)  $\times 5 \times 10^5 \times$  cell sample volume.

**Statistical Analysis.** For comparisons of treatment groups a one-way ANOVA (followed by a Newman-Keuls post hoc test) was performed using tumor measurements taken on the last day recorded. For comparisons of two treatment groups a student's t-test was performed. All statistical analysis was performed using GraphPad Prism software. Statistical significance was achieved when  $P < 0.05$ .

## Results.

*SLC is chemotactic for DC, CD4 and CD8 T-cells in vitro.* Prior to initiation of tumor treatment, we analyzed the effect of SLC on the migration of bone marrow-derived DC and T-cells. As seen in Figure 1, DC were 10 to 100 times more sensitive to SLC, as measured by microchemotaxis, than freshly isolated splenic T-cells, consistent with previous reports (26). Among the major T-cell subsets, significantly more CD4 T-cell migration was seen in response to SLC ( $P < 0.01$ ). In the case of both CD4 and CD8 T-cells,  $> 95\%$  of the migrating cells were of the naive phenotype (as measured by CD62L expression, data not shown). The DC used in these studies were generated from 7-day bone marrow cultures in the presence of GM-CSF and IL-4, but similar chemotactic capabilities were seen from 4-day bone marrow cultures and DC generated in the absence of IL-4 (data not shown).

*Treatment of established tumors with intratumoral injections of SLC.* Since SLC was capable of attracting both DC and naive T-cells in vitro, we addressed the question of whether SLC could promote an anti-tumor effect in vivo. To that end, we established s.c. tumors in B6 mice with an injection of  $3 \times 10^5$  B16-BL6 melanoma cells. We began treatment 6 days after tumor challenge, at a point when palpable tumors were at least  $9 \text{ mm}^2$ . Mice were treated with daily injections of  $3 \mu\text{g}$  of SLC on days 6 through 8 and tumor size was measured. Panel A of Figure 2 shows the results of one representative experiment of 5 performed. When SLC was administered intratumorally, B16-BL6 tumor growth was inhibited by at least 50% of that in mice treated with

vehicle alone ( $P < 0.05$ ). Intratumoral injection of SLC was necessary as tumor growth was not affected by s.c. injections of SLC in the opposite flank. In order to show that this anti-tumor effect of SLC was not strain or tumor type-specific, we treated established breast tumors of the MT901 line in BALB/c mice. Again we found that intratumoral injections of SLC (on days 7 – 9) inhibited growth of established MT901 tumors, while s.c. injection at a distant site had no significant anti-tumor effect ( $P < 0.01$ ; Figure 2B)

To address the dose-dependence of the SLC mediated anti-tumor effect, we treated 6-day established B16 tumors with 3 daily injections of SLC in amounts ranging from 0.1  $\mu\text{g}$  to 25  $\mu\text{g}$ . We found that 0.1  $\mu\text{g}$  had little effect on tumor growth, while doses from 1 – 25  $\mu\text{g}$  resulted in equivalent inhibition of tumor growth ( $P < 0.05$ ; Figure 3). In separate experiments we found no difference between a 3-day and 5-day course of treatment or between 1 or 2 cycles of 5 daily injections that were separated by 2 days (data not shown). Addition of SLC to in vitro cultures of B16-BL6 and MT901 tumor cells had no effect on growth rates (data not shown).

In order to initially address the mechanism operative in the anti-tumor effect of SLC, we treated 6-day established B16-BL6 tumors in mice that had been depleted of either CD4 or CD8 cells 4 days prior to treatment. As seen in Figure 4, depletion of CD8 but not CD4 cells completely eliminated the effect of SLC on tumor growth.

*SLC promotes the recruitment of DC and T-cells to the tumor in vivo.* Since SLC is chemotactic for both T-cells and DC, we hypothesized that intratumoral injections of SLC would elicit migration of these cell types to the tumor site. Mice bearing 6 day established tumors were

treated with intratumoral injections of SLC or PBS for 5 consecutive days. To determine the presence of infiltrating DC, we stained frozen sections for the presence of DEC-205. Tumor from PBS-treated mice contained only rare and focal areas of tumor necrosis at 4 and 7 days after treatment; only isolated and scattered DC were found ( $< 1$  DC per 10 high powered fields) (Table 1). In contrast, tumors from SLC treated mice contained more extensive and frequent zones of necrotic tumor cells (data not shown). Moreover, there were also significantly more infiltrating DC in tumors from SLC treated mice (Table 1). Sections of SLC treated tumors harvested 4 and 7 days after treatment began contained  $6.2 \pm 2.9$  and  $5.9 \pm 1.8$  DC per 10 high powered fields, respectively.

In order to analyze tumor infiltration by T-cell subsets, we harvested tumors on days 2 and 4 of SLC or PBS treatment. Prior to harvest the tumor diameters were measured. Following excision, tumors were enzymatically disaggregated in collagenase to obtain a single cell suspension. We analyzed the tumor samples for the presence of CD4 and CD8 T-cells by FACS analysis. In order to quantify the number of infiltrating lymphocytes unlabelled polystyrene beads (15  $\mu$ m diameter) were added to the samples. Because the tumors were of different sizes, we normalized the number of infiltrating cells to the tumor volume (Figure 5 A & B). After two days of treatment, tumors from SLC-treated mice contained ~5-fold more CD4 and CD8 T-cells than those from PBS-treated animals ( $P < 0.01$ ). Significantly more infiltrating CD4 and CD8 T-cells were also seen after 4 days of treatment ( $P < 0.01$ ). Taken together these data suggested that intratumoral injections of SLC could increase the number of DC and T-cells within the infiltrate of s.c. tumors.

*Genetic Modification of DC to produce SLC.* Our data suggest that the presence of SLC in tumors promotes the migration of DC and T-cells and inhibits tumor growth through a CD8+ T-cell dependent manner. We next determined if alternate routes of delivery of SLC could improve its anti-tumor effect. In other tumor models, intratumoral injections of DC have been shown to promote a T-cell dependent anti-tumor response (16;17). We examined if direct tumoral delivery of SLC via gene-modified DC could improve the anti-tumor effect. To that end we constructed an adenoviral vector containing the gene for SLC.

We used a microchemotaxis assay to determine the levels of SLC produced in the supernatant by gene-modified DC. Supernatants from infected cells were used as the source of chemoattractant in microchemotaxis assays with splenic responder cells. We also performed microchemotaxis using a range (10 – 5,000 ng/ml) of concentrations of recombinant SLC to generate a standard curve for T-cell chemotaxis. From the standard curve we could determine the concentration of chemokine present in DC cultured supernatants. The standard curve used in determining SLC concentrations was generated by analyzing the migration of CD4+ cells in the splenocyte responders. However, similar values were obtained using a standard curve generated from migrating CD8+ cells or by total migrating lymphocytes (data not shown). As shown in Figure 6A, which represents the data from 9 separate experiments, ~ 750 ng of SLC was produced within 18 hours by SLC gene-modified DC. SLC was detected in 24 hour culture supernatants 3 days after infection, suggesting that gene expression endured for at least this period of time (Figure 6B). Genetic modification of DC with adenoviral vectors resulted in a modest up-regulation of the T-cell co-stimulatory receptors CD80 and CD86 (data not shown).

The amount of chemotactic activity in cultured supernatants from SLC gene-modified DC was > 10 fold more than that elicited by GFP expressing DC (Figure 6A). Cultured supernatants from GFP gene-modified DC promoted microchemotaxis of T-cells to the same extent as that of cultured supernatants from the D5 variant of the B16 cell line (data not shown). This finding suggests that although DC produce chemokines (44), including the CCR7 agonist ELC, they did not appear to be present in large amounts in cultured supernatants.

*Intratumoral injections of SLC expressing DC promote a potent antitumor effect.* We used SLC gene-modified DC to treat 6-day established B16 tumors. Mice received 3 intratumoral injections of SLC (or GFP) gene-modified DC on days 6, 9 and 13. Another cohort of mice also received daily injections of recombinant SLC for 5 consecutive days beginning on day 6. Mice receiving GFP gene-modified DC showed some inhibition in tumor growth (Figure 7), consistent with previous reports (16;17). The antitumor effect of the GFP expressing DC was slightly less than that elicited by recombinant SLC. However, intratumoral injections of SLC gene-modified DC elicited an anti-tumor effect that was significantly greater than that elicited by either the GFP expressing DC or SLC alone ( $P < 0.01$ ; Figure 7).

*Tumor lysate-pulsed, SLC gene-modified DC elicit systemic anti-tumor immunity.* We next determined if treatment of established B16-BL6 tumors with tumor lysate-pulsed DC could be improved by genetically modifying the DC to express SLC. Pulsing of DC with tumor lysate did not significantly affect the levels of SLC produced by gene modified DC (data not shown). We immunized mice bearing 6-day established tumors with DC expressing SLC (or GFP) and pulsed

with B16-BL6 tumor lysate contralaterally to the site of growing tumors. Figure 8 shows that while GFP gene-modified DC were ineffective in reducing the growth of s.c. tumors, SLC expressing DC were able to mediate a significant anti-tumor response. This response was dependent upon presentation of tumor antigen(s) by the DC, as unpulsed SLC gene-modified DC were unable to elicit an anti-tumor response when administered at a site distal from the tumor (Figure 8).

One possible explanation for the enhanced effect of SLC gene modification on tumor lysate-pulsed DC immunizations is enhanced recruitment of host derived T-cells *in vivo*. Since the vast majority of DC remain at the s.c. immunization site 24 hours post injection (45;46), it is possible that T-cells would migrate to skin sites containing SLC expressing DC. To determine the influx of T-cells into DC skin injection sites, we immunized mice s.c. with B16-BL6 lysate-pulsed DC expressing either GFP or SLC and harvested skin samples 3 days later. Following enzymatic disaggregation, we analyzed these samples for the presence of CD4<sup>+</sup> and CD8<sup>+</sup> cells by FACS. As shown in Figure 9 we found that SLC expressing DC attracted ~2 – 3 fold more CD4 and CD8 T-cells to the injection site at both time points tested ( $P < 0.05$ ). These data suggest that the improved adjuvanticity of DC resulting from expression of SLC may be due, in part, to increased migration of T-cells to the site of immunization.

## Discussion

In this study we tested the anti-tumor properties of SLC in three treatment regimens. Using direct tumoral administration of recombinant protein, we found that SLC could inhibit the growth of the B16 melanoma and the MT901 mammary adenocarcinoma. SLC caused a marked influx of DC, CD4+ and CD8+ T-cells into the tumor mass. In vivo depletion of CD8+ T-cells eradicated the anti-tumor effect of SLC. Using an adenoviral vector encoding SLC, we were able to generate SLC expressing DC derived from bone marrow progenitors. When given intratumorally, these SLC gene-modified DC could elicit an anti-tumor effect that was significantly better than either the recombinant protein or GFP gene modified DC. Finally, we found that SLC gene-modification substantially improved the adjuvanticity of tumor lysate-pulsed DC against the poorly immunogenic B16-BL6 melanoma and could attract T-cells to the s.c. immunization site. Due to its poor immunogenicity, tumors of B16-BL6 origin are refractory to treatments that would ordinarily lead to tumor regression of weakly immunogenic tumor lines (15;16), particularly when tumor burden is high (e.g. 6-day s.c. tumors).

The receptor for SLC, CCR7, is expressed on both naïve T-cells and DC, suggesting that it plays an important role in T-cell activation in peripheral lymphoid organs where the chemokine is expressed (20-23). This is underscored by the fact that both SLC deficient (*plt*) and CCR7<sup>-/-</sup> mice have reduced responses to antigenic stimulation (29;30). Because we found evidence of both DC and T-cell migration in tumors treated with SLC, it is possible that the emigrated T-cells are being primed in the tumor by infiltrating DC that have taken up necrotic tumor cells (47). Furthermore, since mature DC may be able to activate CD8+ T-cells in the absence of CD4+ T-cell help (48), intratumoral priming of CTL may explain why SLC treatment



is efficacious even in the absence of CD4<sup>+</sup> cells (Figure 4). However, we cannot rule out the possibility that mature DC attracted to the tumor by the presence of SLC take up TAA and migrate to the draining node where they activate CTL. We are currently investigating the phenotype and function of the tumor-infiltrating T-cells as well as the stimulatory capacity of emigrating DC.

In a recent report, Sharma et al. (49) showed that recombinant SLC could inhibit the growth of 5-day established tumors in mice. In this particular study, SLC mediated anti-tumor immunity was not elicited in either CD4 or CD8 deficient mice. The dependence upon CD4<sup>+</sup> T-cells contrasts with our data using antibody depletion (Figure 4). These disparate results could be explained, in part, by the experimental systems used to determine subset contributions (subset deficient mice for Sharma et al. v. antibody depletion here) or by the tumor models employed (3LL and LC12 lung cancers v. B16 melanoma). Sharma et al. (49) also reported an increase in tumor infiltration by DC and T-cells as a result of SLC treatment, which is in agreement with our data (Table 1 and Figure 5).

Direct tumoral administration of other recombinant chemokines, namely IP-10 and Mig, has been shown to inhibit tumor growth as well (50-52). In these cases, the anti-tumor response was mediated by the anti-angiogenic properties of these chemokines. IP-10 and Mig can mediate the anti-tumor effect of IL-12 (50;53), which may explain, in part, the increased anti-tumor effect elicited by IL-12 gene modified DC (16). SLC has been shown to bind to CXCR3, the receptor for both IP-10 and Mig, and to exert an angiostatic activity in vivo (54). However, because the anti-tumor effect of SLC was eliminated in mice depleted of CD8<sup>+</sup> T-cells, it is unlikely that SLC mediated its anti-tumor effect via direct inhibition of angiogenesis. However, it remains a

possibility that SLC can indirectly affects tumor vasculature via recruitment of DC and T-cells that produce angiostatic agents such as IP-10 and Mig.

Anti-tumor therapies based on chemokine gene transfer and expression have utilized chemokine-transfected tumor cells, adenoviral gene delivery to tumors and gene-modified DC (14;15;55-59). Previously, we reported that tumor cells stably expressing the CXC chemokine RANTES failed to grow in immunocompetent hosts (55). Similarly to intratumoral injections of SLC, the anti-tumor effect elicited by RANTES secreting tumor cells was dependent upon CD8<sup>+</sup> T-cells. However, we were unable to detect T-cell or DC migration in response to RANTES in vitro (data not shown). Furthermore, RANTES secreting tumors were ineffective as a treatment against established tumors (55). More recently, it was reported that tumor cells stably expressing ELC, another ligand for CCR7, also fail to grow in immunocompetent hosts (59). In contrast to our work with SLC, the anti-tumor response reported by this group was dependent upon NK and CD4<sup>+</sup> cells but did not involve CD8<sup>+</sup> cells. Lymphotactin (Lptn), a C chemokine, has been shown to enhance an anti-tumor effect in two gene therapy models (14;15;60). Immunization of tumor bearing mice with irradiated tumors containing Lptn secreting cells had little effect on tumor growth, but resulted in dramatic reduction in tumor growth when combined with IL-2 secreting cells (60). DC genetically-modified to express Lptn and pulsed with either peptides derived from tumor antigens or tumor RNA, triggered a stronger anti-tumor response than control gene modified DC (14;15). However, the receptor for Lptn is not expressed on naive T-cells (61), suggesting that the effect of Lptn gene expression depends on already activated T-cells. Since CCR7 is found on naive T-cells, our results are consistent with a model in which SLC enhances the priming of naive T-cells through antigen presenting DC.

Cytokine and chemokine gene-modified DC promote stronger anti-tumor responses than their control gene-modified counterparts, regardless of whether the DC are delivered intratumorally or pulsed with tumor antigens and administered at a distal site (13-17;62). Here, we show that SLC expressing DC are superior to GFP gene-modified DC in both treatment regimens. Gene-modified DC express substantial amounts of SLC ( $\sim 750 \text{ ng}/1 \times 10^6 \text{ cells}/18 \text{ hr}$ ) and the adenoviral vector has no detrimental effect on DC phenotype. To our knowledge this is the first report of genetic-modification of DC to express a chemokine selective for naïve T-cells. Of note, unmodified and control modified DC cultured supernatants resulted in minimal migration of T-cells, to an extent equivalent to those from an unmodified tumor cell line. One interpretation of these data is that although DC express the genes for several chemokines, including ELC, they do not express significant amounts of the protein (44). Another possibility is that DC cultured in vitro, remove the secreted ELC via CCR7 expressed on their surface. In this model, it is possible that DC also bound and removed the secreted SLC, but due to high expression levels, detectable amounts remained in culture.

When given intratumorally, SLC expressing DC reduced tumor growth of established B16 melanoma tumors to a greater extent than either DC alone or SLC alone. Since addition of recombinant SLC resulted in the infiltration of CD4 and CD8 T-cells (along with DC), it is likely that injection of SLC expressing DC also resulted in T-cell infiltration and possible activation of T-cells by the injected DC (or by endogenous DC attracted to the tumor by SLC). It is also possible that SLC expressing DC acquired TAA and migrated to the draining lymph node to enhance T-cell priming. Our future studies will determine the migratory capacities of SLC gene-modified DC in vivo. Another explanation for the enhanced effect of SLC expressing DC could

be due to the bioavailability of the protein in vivo. Recombinant SLC was given intratumorally once daily for 5 days while the DC were given three times over the course of 7 days. Since DC expressed high levels of SLC in vitro for at least 3 days, it is possible that a therapeutically effective dose of SLC in the tumor (i.e.  $> 0.1 \mu\text{g}$ ) was maintained longer by the addition of SLC expressing cells. However, the kinetics and levels of SLC gene expression in vivo by adenovirus-infected DC have yet to be determined.

We were also able to achieve efficacious treatment of established tumors by immunization with lysate-pulsed, SLC-expressing DC while GFP gene-modified DC were ineffective as an adjuvant in this tumor model. To our knowledge this is the first report combining chemokine gene-modification of DC with tumor lysate pulsing to generate a therapeutically effective cancer treatment. One possible mechanism by which SLC enhanced the immunogenicity of DC based vaccines was by the recruitment of T-cells to the immunization site. It has been shown in both mice and humans that the vast majority of DC injected s.c. remain in the injection site and do not reach the draining lymph node (45;46). Here we showed that SLC expressing DC could recruit T-cells to the immunization site. It is possible that the tumor lysate-pulsed DC activated TAA specific T-cells within the migratory population locally. If indeed some T-cells had become activated, it is not likely that they remained in the s.c. area for extended periods. T-cell migration to the skin in response to D5 cells expressing ELC, which binds to CCR7, occurred at 48 and 72 hours post immunization but were no longer present 4 days after injection (Kirk, Giedlin and Mulé, unpublished results). Furthermore, T-cells have been shown to lose expression of CCR7

following activation (63), suggesting that, once activated, TAA specific T-cells would no longer be expected to be retained in the immunization site by the SLC expressing DC.

While our results do not show unequivocally that anti-tumor immunity is triggered by the DC residing in the injection site, they do suggest that SLC expression may increase the effective number of DC (i.e. those that prime naïve T-cells) present in each immunization. Future studies using mice lacking peripheral lymph nodes should address the question of whether SLC expressing DC can prime an immune response without migration to lymph nodes. SLC gene-modification may obviate the need for intranodal delivery of DC presently used in some clinical applications (10). Comparison of the route of delivery (e.g. s.c. v. i.v. or i.p.) of SLC gene-modified DC will further address the mechanisms behind the enhanced adjuvanticity of these cells. While this study utilized a first generation adenovirus, use of "gutted" adenoviral vectors (38), which allow for the incorporation of large amounts of cDNA, will allow for the gene transfer of multiple cytokine and/or chemokine genes within a single vector. These newer generation vectors are also believed to be less immunogenic than earlier versions (64), lessening the possibility of gene modified DC inducing anti-viral immunity, which has been reported by others (65;66). Our data demonstrate that SLC may be utilized as a therapeutic agent for the treatment of established tumors as both a stand alone biotherapeutic and a gene therapy in conjunction with DC based treatments.

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## Figure Legends

**Figure 1.** Chemotactic response of dendritic cells and T-cell subsets towards SLC. DC ( $2.5 \times 10^5$ /sample) derived from 7 day bone marrow cultures or splenocytes ( $1 \times 10^6$ /sample) from B6 mice were placed in the upper chamber of 6.5 mm transwell inserts (5  $\mu$ m pore size) with recombinant SLC added to the lower chambers in the indicated concentrations. Following a 2 hour incubation at 37°C,  $10^4$  polystyrene beads were added to each well and the samples stained for DC markers (MHC II and CD86) or for CD4 and CD8. The number of migrating cells in each sample was calculated as described in Material and Methods. The migrating samples were compared to input samples that did not involve microchemotaxis and the data is reported as % input migrating cells for DC (open bars), CD4 (hatched bars) and CD8 (black bars). \* =  $P < 0.01$  for CD4 v. CD8 by student's t-test.

**Figure 2.** Antitumor effect of SLC. (A) B6 mice were injected with  $3 \times 10^5$  B16-BL6 melanoma cells in the s.c. right flank. SLC ( $\Delta$ ) was given intratumorally at 3  $\mu$ g per dose in 50  $\mu$ l PBS (with 0.05% normal mouse serum) on days 6 – 8. Control groups received intratumoral injections of PBS (with 0.05% normal mouse serum) alone ( $\square$ ) or SLC ( $\blacktriangledown$ ) in a distal site. Tumors were measured twice weekly by measuring the longest perpendicular diameters and presented as tumor area in  $\text{mm}^2$ . \* =  $P < 0.05$  for intratumoral SLC v. other groups. (B) BALB/c mice bearing 7-day s.c. tumors of the MT901 mammary adenocarcinoma ( $1 \times 10^6$  tumor inoculum) were treated with intratumoral injections of SLC as described above from days 7 – 9. Control groups and tumor

measurements were as those in (A). \*\* =  $P < 0.01$  for intratumoral SLC v. other groups. These data are representative of four other experiments with B16-BL6 and two other experiments with MT901, all with similar results.

**Figure 3.** Dose dependence of anti-tumor effect of SLC. Mice with 6-day established s.c. tumors of the B16-BL6 melanoma ( $1 \times 10^5$  tumor cell inoculum), were treated with 3 daily injections of SLC at the indicated doses. Tumors were measured twice weekly and presented as tumor size in  $\text{mm}^2$ . \* =  $P < 0.05$  for SLC doses of 1 – 25  $\mu\text{g}$  v. PBS. These data are representative of two similar experiments.

**Figure 4.** Depletion of CD8<sup>+</sup> cells abrogates the anti-tumor effect of SLC. B6 mice were inoculated with  $1 \times 10^5$  B16 cells in the right flank. Antibodies to CD4 (●) and CD8 (○) were added 4 and 3 days prior to treatment as described in Materials and Methods. Antibodies were also added 3, 7 and 10 days after treatment with SLC (3 $\mu\text{g}/\text{dose}$ ) began. Control mice treated with SLC ( $\Delta$ ) or PBS ( $\square$ ) received an equivalent amount of irrelevant isotype-matched antibody. Mice were treated from days 6 – 10 and tumor size was measured as described in Figure 2. These data are representative of two experiments with similar results.

**Figure 5.** Intratumoral injection of SLC elicits infiltration of host derived T-cells. (A) & (B) Tumors were harvested 2, 4 and 7 days after beginning treatment with either SLC ( $\Delta$ ) or vehicle ( $\square$ ), digested with collagenase and analyzed for the presence of CD4 (A) and CD8 (B) by FACS.



Quantitation of T-cell infiltration was determined by the addition of polystyrene beads (final concentration  $5 \times 10^5/\text{ml}$ ) and normalized to tumor volume as describes in the materials and methods. Data presented as the mean  $\pm$  SEM from 3 – 4 mice/group (except for 7 days in which 2 mice/group were tested). \* =  $P < 0.01$  by Student's t-test.

**Figure 6.** Genetic Modification of DC to produce SLC. (A) DC were harvested from 4 – 6 day old bone marrow cultures and infected with adenoviral vectors encoding for either SLC or GFP as described in Materials and Methods. Supernatants were harvested 18 hours post infection and were used in the bottom chamber of a 24 well plate microchemotaxis assay; CD4+ T-cells from splenocytes served as responders. Recombinant SLC ( $10 - 5,000 \text{ ng/ml}$ ) was used to generate a standard curve from which the effective concentration of SLC in the cultured supernatants was determined. Duplicate to quadruplicate samples were run for each supernatant tested. Data are presented as the mean ( $\pm$  SEM) amount (in ng) of SLC produced in 18 hours by  $1 \times 10^6$  cells and is cumulative of 9 separate infections. (B) Cultured supernatants were harvested every 24 hours for 3 days following infection of DC with adenoviral vectors encoding SLC (●) or GFP (○) and used in microchemotaxis assays as described in (A). Supernatants were also taken from uninfected DC (□). Values are presented as the effective amount (in ng) of SLC produced in 24 hours by  $1 \times 10^6$  cells.

**Figure 7.** Direct tumoral administration of SLC expressing DC inhibits growth of established tumors. Mice were injected s.c. with  $5 \times 10^4$  B16 cells.  $5 \times 10^5$  DC infected 18 hours prior with

adenovirus encoding SLC (●) or GFP (○) were given intratumorally on days 6, 9 and 13.

Control mice received HBSS alone (□) and another cohort received daily injections of 3 μg recombinant SLC (Δ) on days 6 – 10. Tumor size was measured twice weekly and is presented as tumor area in mm<sup>2</sup>. This experiment was performed twice with identical results. \* = P < 0.01 for DC-SLC v. SLC or DC-GFP.

**Figure 8.** Immunization with tumor lysate-pulsed SLC gene-modified DC elicits anti-tumor effect. Mice bearing 6-day s.c. B16-BL6 tumors in the right flank were immunized in the left flank twice on days 6 and 13 with  $5 \times 10^5$  DC that had been infected with adenovirus encoding SLC (●) or GFP (○) and pulsed for 18 hours with B16 cell lysate. Control mice received injections of HBSS alone (□). Tumor size was measured as in Figure 7.

**Figure 9.** CD4+ and CD8+ T-cells migrate to the injection sites containing SLC expressing DC. Mice were injected s.c. with  $1 \times 10^6$  tumor lysate-pulsed DC expressing either GFP (open bars) or SLC (hatched bars). Three days after injection, 1.5 x 1.5 cm sections of skin were harvested, minced and digested in collagenase, DNase I and hyaluronidase to obtain a single cell suspension ( $\sim 1 \times 10^6$  cells/ml). Polystyrene beads ( $C_f = 5 \times 10^5$  beads/ml) were added to enumerate migrating T-cells. T-cells subsets were analyzed by FACS. Data are presented as the mean ( $\pm$  SEM) number of migrating cells from 4 (GFP) and 5 mice (SLC). \* = P < 0.05 by students t-test

**Table I.** Enumeration of DC<sup>a</sup> infiltrating B16-BL6 tumors by SLC treatment.

Treatment <sup>b</sup>	Day 4 <sup>c</sup>	Day 7 <sup>c</sup>
PBS	< 1	< 1
SLC	6.2 ± 2.9	5.9 ± 1.8

<sup>a</sup> DC were visualized by DEC-205 staining as described in Materials and Methods

<sup>b</sup> Mice bearing 6-day tumors s.c.; PBS or SLC (3 µg) was administered daily intratumorally for 5 consecutive days; tumors harvested 4 and 7 days post treatment

<sup>c</sup> # DC per 10 high powered fields

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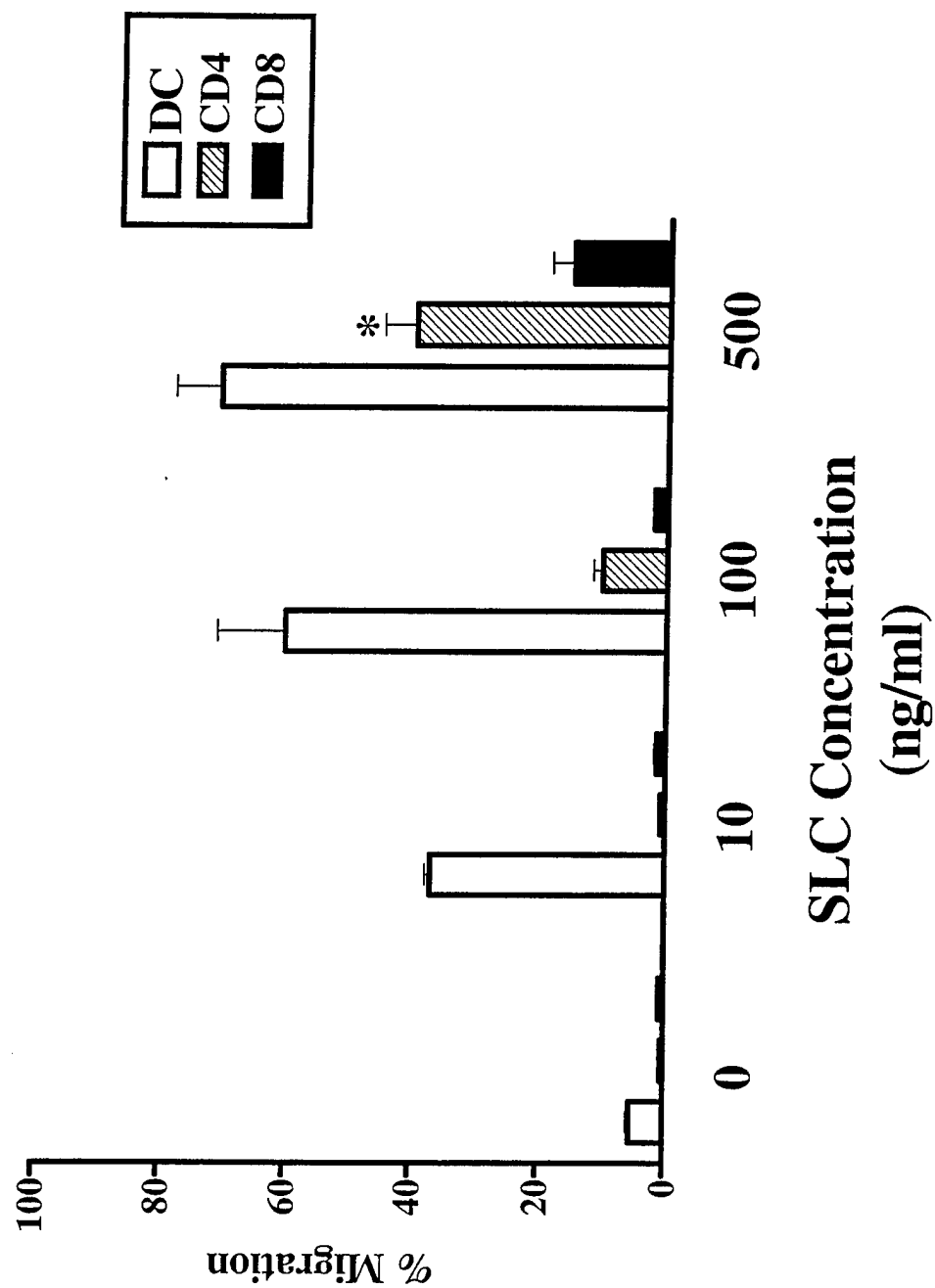
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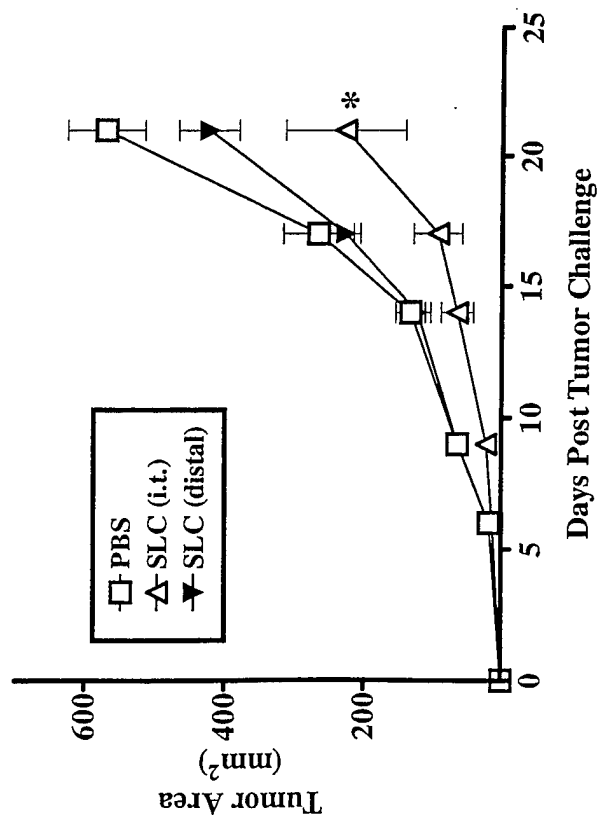
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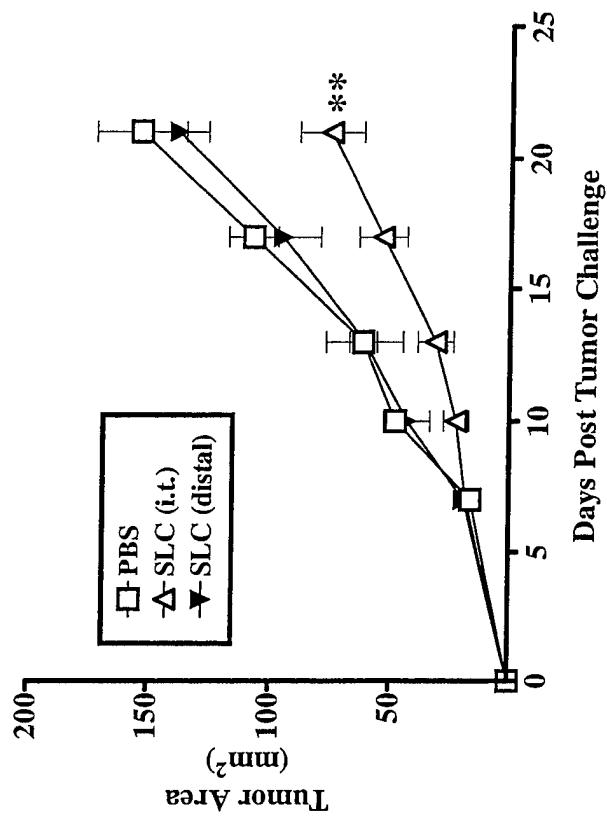


Kirk, *et al.* Figure 1

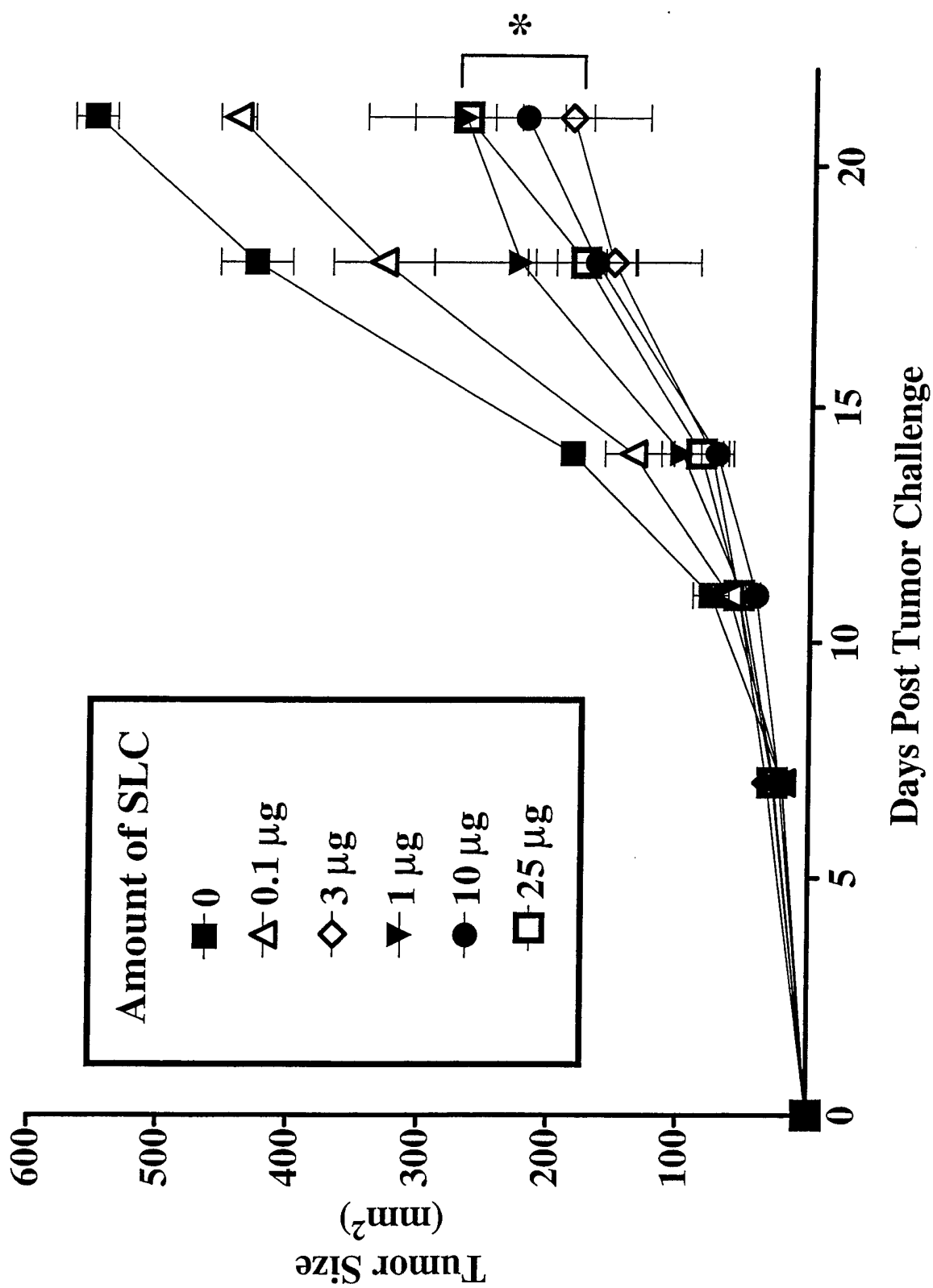
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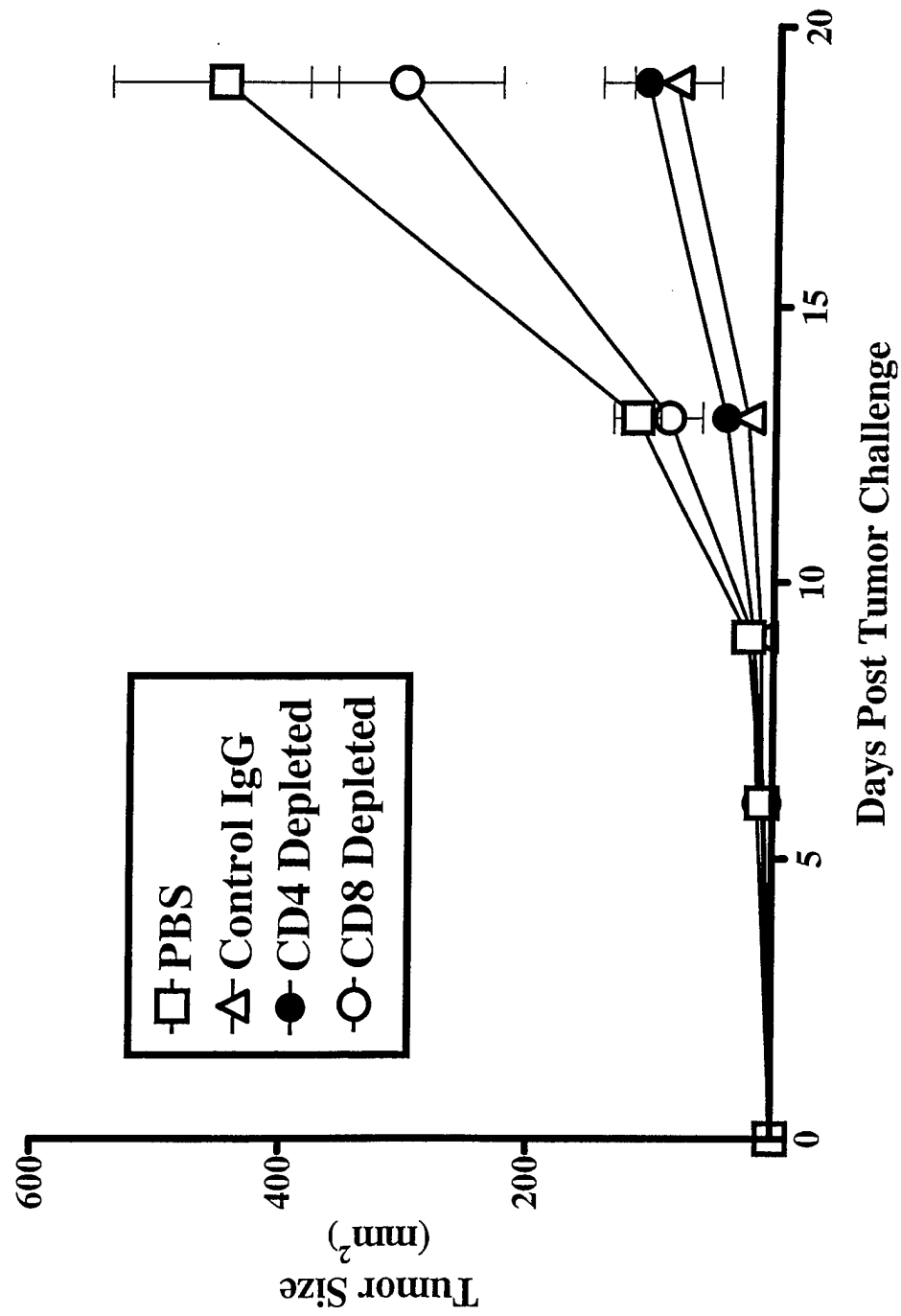


B



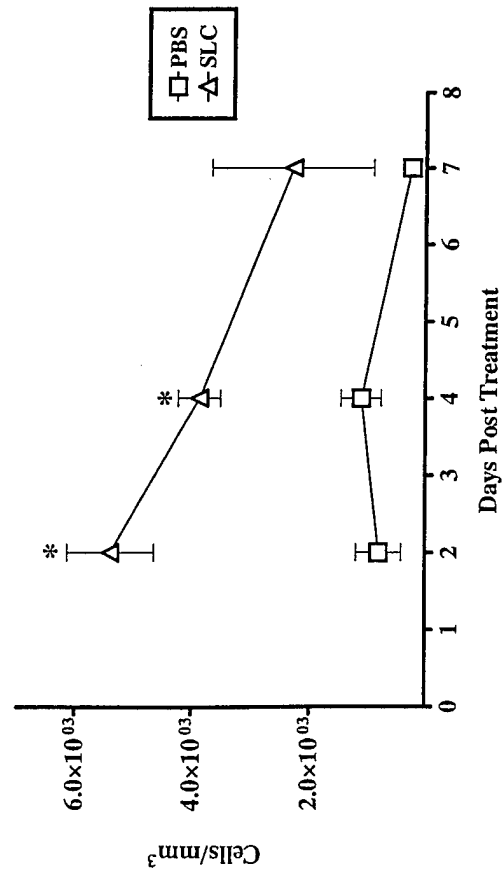




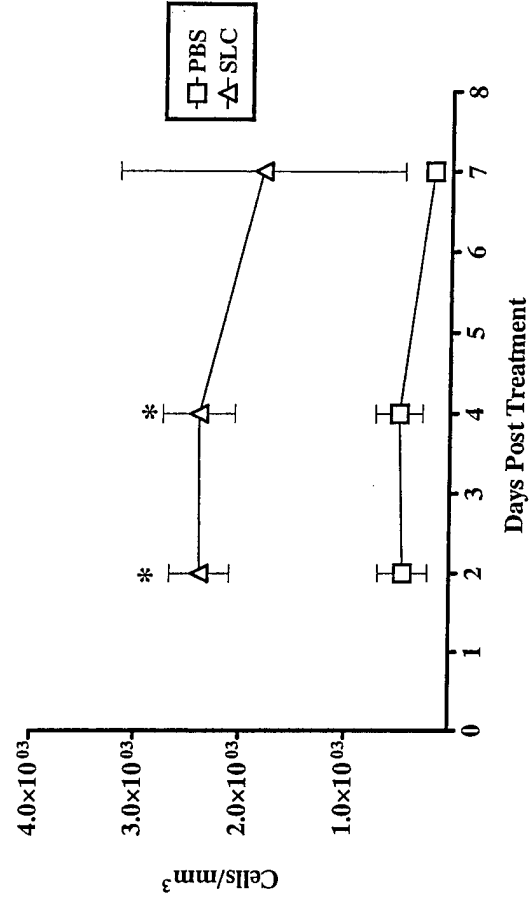


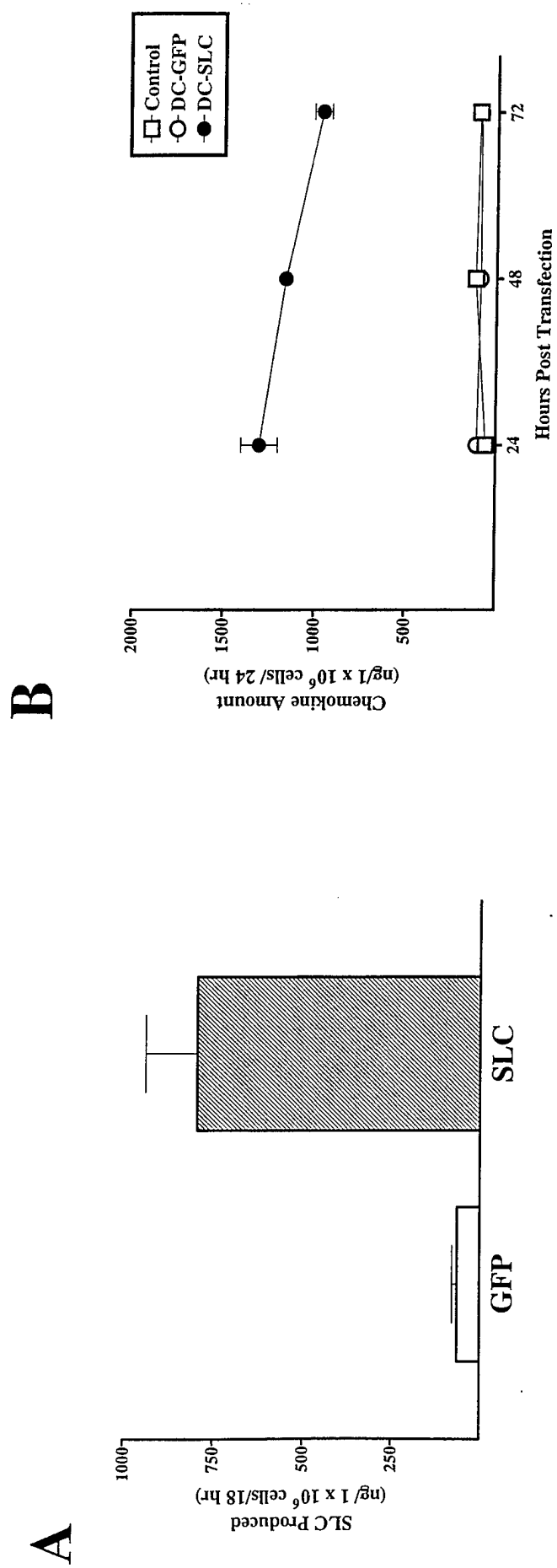
Kirk *et al.* Figure 4

A

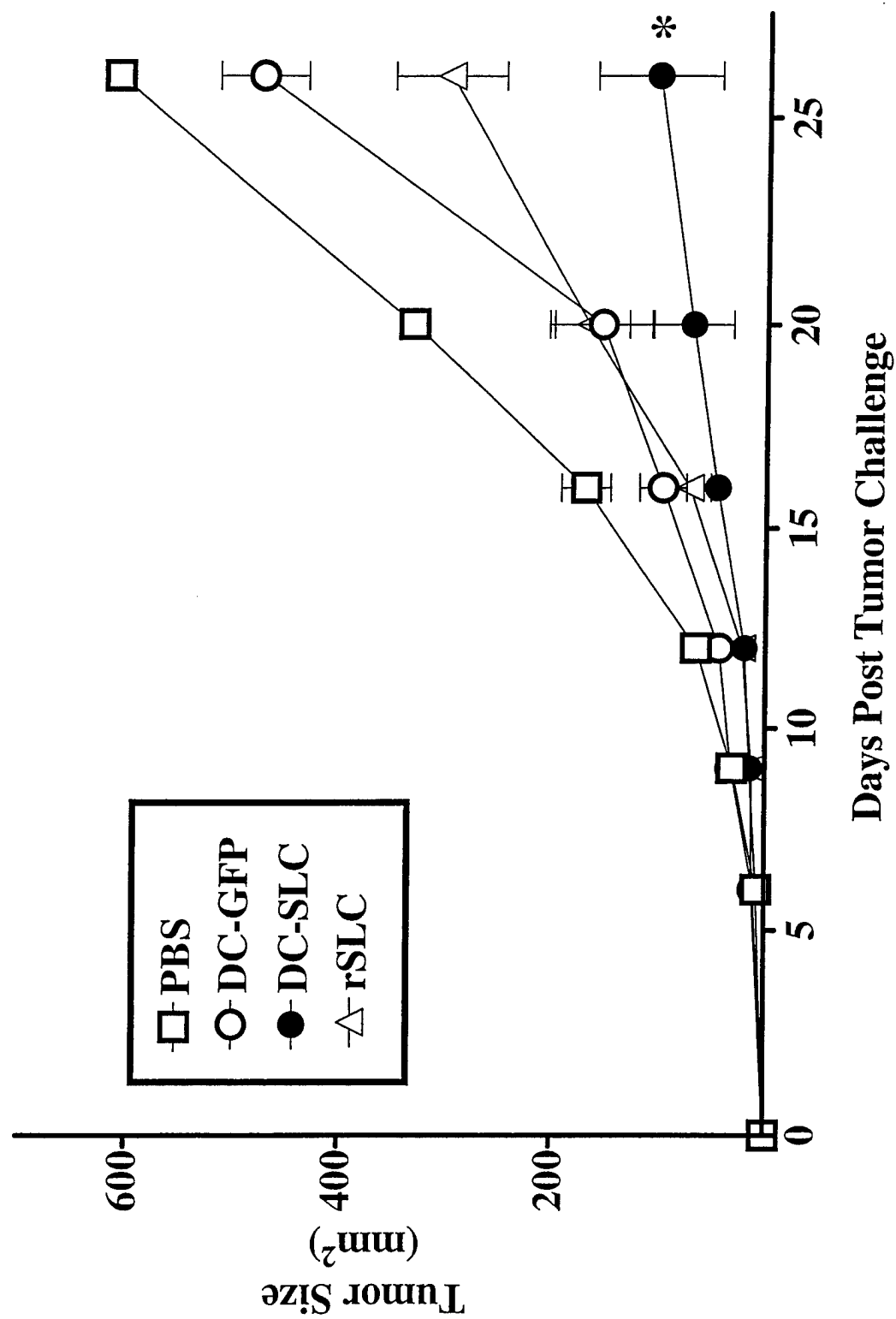


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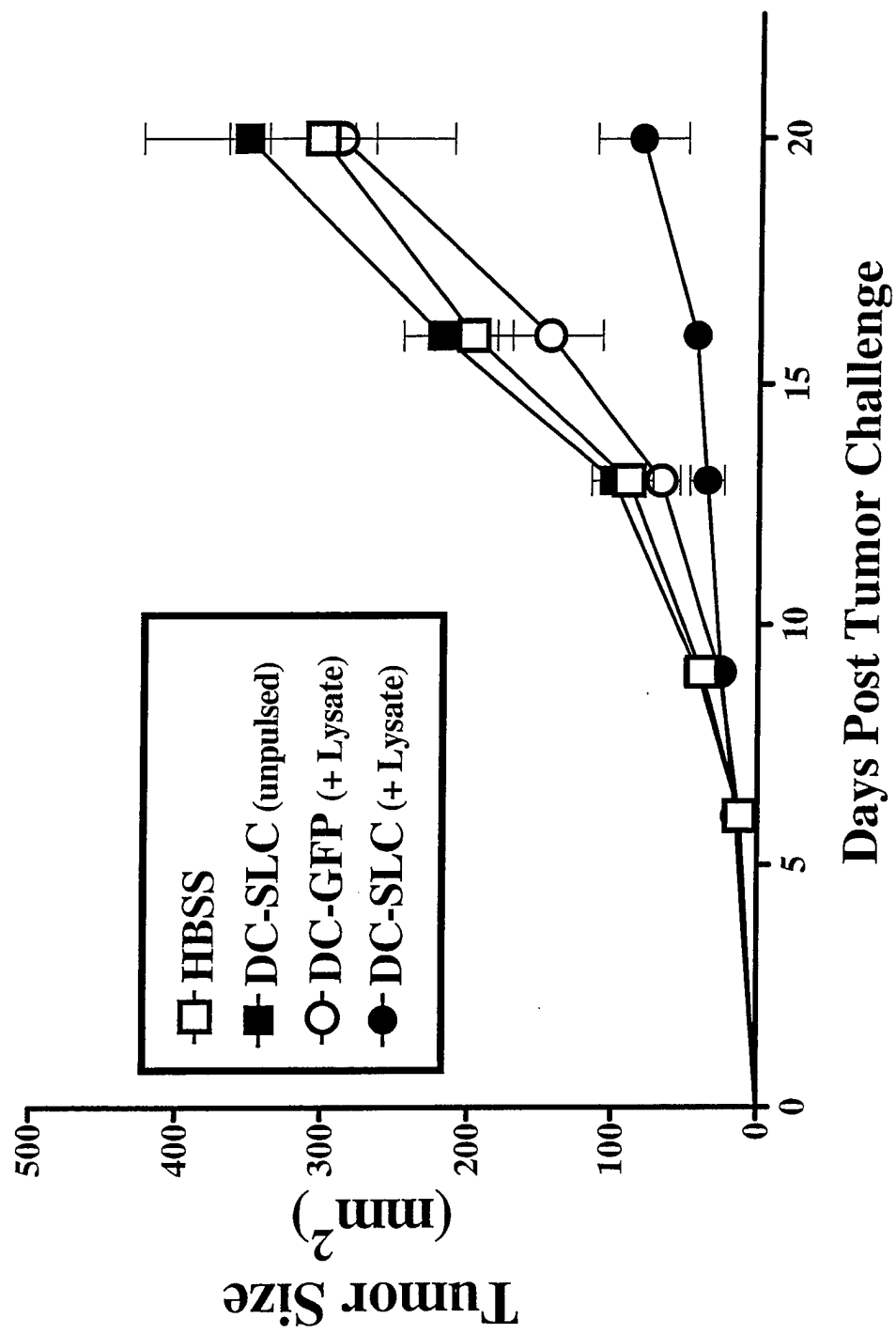




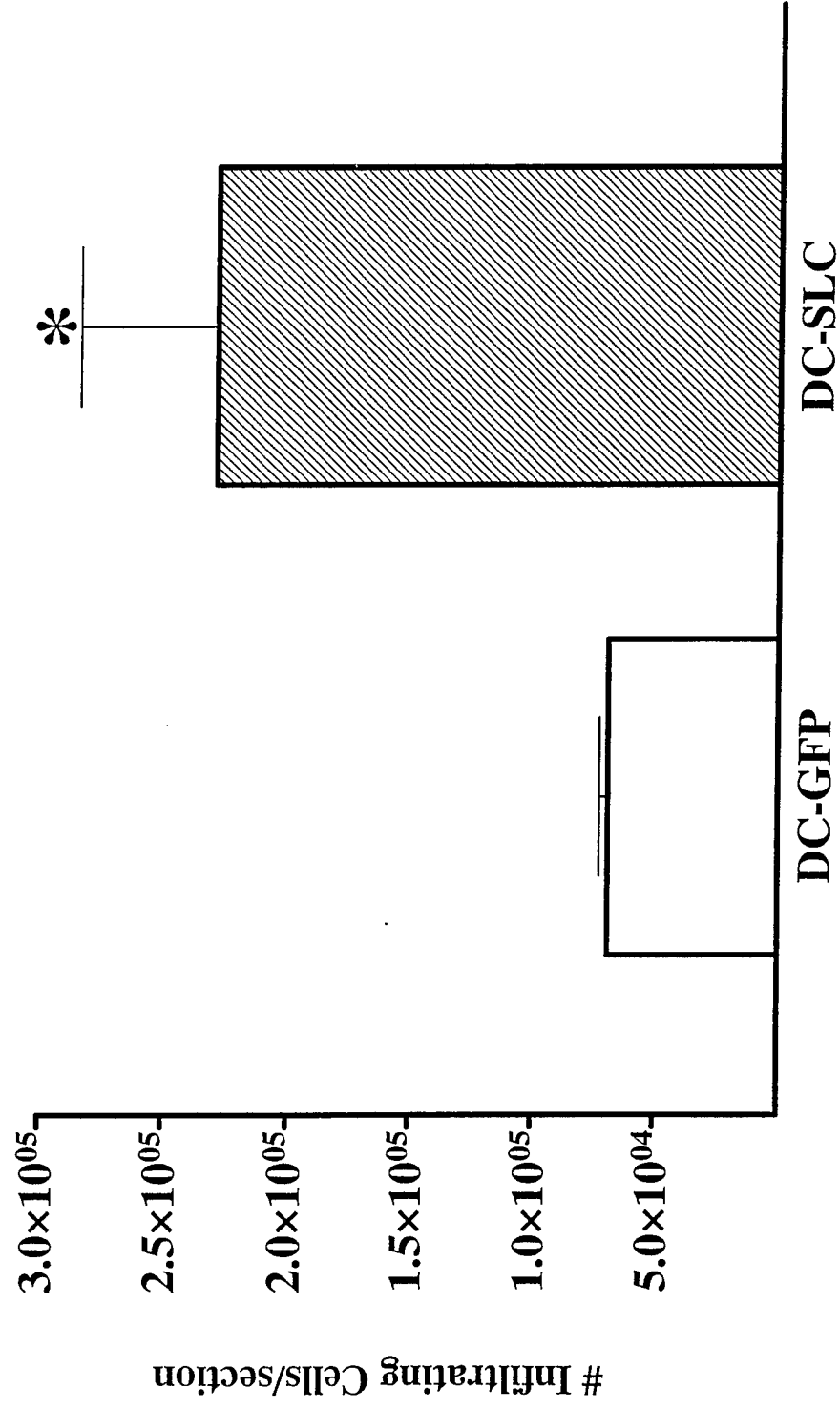
Kirk et al. Figure 6



Kirk *et al.* Figure 7



Kirk *et al.* Figure 8



Kirk *et al.* Figure 9